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Innovative gold complexes with CN group as anticancer agents – possible mechanisms of action

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ABSTRACT

Several gold(I) and gold(III) compounds described so far have significant antiproliferative effects *in vitro* against selected tumor cell lines. Gold (III) has the same electronic configuration as platinum(II); moreover, some gold(III) complexes have the same square-planar structure as cisplatin. In addition, the compounds are water-soluble and display low toxicity. The disadvantage of gold(III) derivatives is low stability in physiological conditions that reduces their applications. Thus, an invention of novel gold complexes and characterization of their mechanisms of action is extremely urgent. Here, we invented and synthetized a library of innovative gold(III)-based drug-candidates. Those metallodrugs were synthetized according to the following formula: $[Au(CN)_n]_m^{(m-p)n} \cdot (ClO_4)_m$, where: "m" is a number from 3 to 6, "n" is from 1 to 10, "p" is from 1 to 3 and "r" is from 0.1 to 2. Initial physicochemical studies showed that novel gold(III) complexes were stable in water, blood and lymph. Within this work, our team aimed to critically discuss the mechanisms of action of new gold(III) complexes in cancer treatment, in particular the complex TGS121: $[Au(CN)_4]_2(ClO_2)Na$, based on the structure of the new compound and available experimental evidence. Great antiproliferative properties were observed against Ha-Ras-transfected malignant fibroblasts. The IC50 values for parental normal fibroblasts were significantly lower than in transfected cells. TGS121 turned out to be high selective for malignant cells in comparison to normal cells. The analysis of the mole-cular structure suggested that TGS121 could produce a biological effect through a variety of molecular mechanisms. These results make the novel gold(III) complex attractive for further evaluation as an anticancer agent.

Keywords: bioorganometallics; biphenyl; cancer; gold chelates; gold (III) complexes, TGS121

INTRODUCTION

Inorganic chemistry parallel to organic chemistry is beginning to have an increasing impact on medicine. Cisplatin, a platinum(II) complex, was the first metal-based agent to enter into worldwide clinical use for the treatment of cancer. The discovery of the anticancer effects of cisplatin, around 1965 (Rosenberg, 1985), suggested that platinum and non-platinum metal-based compounds might complement the role of organic anti-cancer drugs (Galluzzi, 2014). This successful platinum-based complex is effective at inhibiting the activity of cancer cells by producing a direct lesion on DNA. This non-selective, DNA-targeting mechanism produces several side effects, such as cardiotoxicity, nephrotoxicity and neurotoxicity (Rezaee 2017; Wang, 2013; Altoum, 2017). Currently other antitumor, metal-based complexes are studied, like ruthenium, gold and titanium (Muhammad, 2014). Amongst them, complexes of gold in oxidation states +I and +III have attracted particular attention. Several studies highlighted that the binding affinity of gold complexes for the DNA was relatively low, indicating that gold compounds have a different mechanism of action than cisplatin (Casini, 2008; Casini, 2010; Mirabelli, 1986). In 1985, auranofin, a gold(I) complex, was approved by the U.S. Food and Drug Administration (FDA) as a therapeutic agent to treat rheumatoid arthritis. Moreover, auranofin demonstrated promising results for the treatment of various malignancies including: leukemia, lung, ovarian, gastric and pancreas cancer 9 (Furst, 1983; Fiskus, 2014; Park, 2014; Zou, 2015; Rios Perez, 2019; Xiaobo, 2016). Concurrently, gold(III) compounds, due to the same squareplanar structure to cisplatin and the fact that gold in oxidation states +III is isoelectronic to platinum in +II, have been qualified as excellent candidates for potential anticancer drugs. The oxidation state +III typically bears a pronounced oxidizing character, unless the gold(III) center is stabilized by an appropriate set of ligands (Gabbiani, 2011). Thus, clinical usefulness of gold(III) compounds was found to be limited, because Au(III) was reduced to

Au(I) or even Au(0) under physiological conditions. This disadvantage slowed down the investigation of gold(III) derivatives in anti-cancer treatment. In mid-1990, Parish et al. (Parish, 1996) invented the first gold(III) complexes with acceptable solution stability and with encouraging results of *in vitro* cytotoxicity toward selected human tumor cell lines. Subsequently, several other classes of cytotoxic gold(III) compounds were developed in many laboratories all over the world, but none of them have been approved for clinical use (Casini, 2009).

Some initial indications concerning the possible mechanisms of action of gold(III) compounds were obtained. As mentioned above, it seems unlikely that all gold compounds work analogously to cisplatin. In fact, interactions with proteins may play key roles in the mechanism of action and in the toxic effects of these antitumor metal complexes (Mirabelli, 1986; Gabbiani, 2011). Nevertheless, the molecular targets of antiproliferative gold compounds are still largely unknown and a subject of intense research and debate. Casini et al. (Casini, 2009) evaluated the anticancer properties of a group of gold(III) derivatives against a panel of 36 human tumor cell lines using a systematic screening strategy. It was observed that the antiproliferative properties essentially rely on a variety of distinct molecular mechanisms. In particular, possible targets for the investigated gold compounds were proposed, e.g., thioredoxin reductase, protein kinase C, histone deacetylase, and proteasome. On the other hand, Barnard et al. (Barnard, 2007) advanced a hypothesis that cytotoxic gold compounds, in particular gold(I) compounds, produce their biological effects mainly through a direct antimitochondrial mechanism. No doubt, the same mechanism cannot be excluded for gold in oxidation states +III.

Analysis of the state of the art in the area of metallodrugs in anticancer treatment, particularly based on gold, clearly shows that there is still room for improvement and strongly encourages further exploration of the field. An ideal gold-based chemotherapeutic agent should be well-soluble in water, stable in the blood and lymph and efficient at a relatively low dose. The biggest disadvantage in the clinical application of currently available gold complexes is their breakdown in the blood and lymph, decreasing the therapeutic efficacy. This adverse appearance leads to the requirement of the use of much higher doses, which in turn causes increased risk of gold accumulation in selected organs and side effects. Consequently, four new, innovative gold(III)-based drugs were synthesized by the following formula: $[Au\ (CN)_n]_m^{(m-p)n} \cdot (ClO_4)_m$, where: "m" is a number from 3 to 6, "n" is from 1 to 10, "p" is from 1 to 3 and "r" is from 0.1 to 2. Here, we describe the composition and properties of one compound, termed TGS121, synthetized using innovative mono ion technology. This novel method results in elevated bioavailability through the prevention of formation of large water-soluble gold clusters. These clusters are characterized by the presence of a metal – metal bond (Au-Au) and have the structure of crystallographic lattices that are not able to freely pass through the cell membrane. The novel gold(III) complex has no Au – Au bonds, which provides better delivery and can reduce the compound dosage in biological applications. Initial physicochemical studies showed that the novel gold(III) complex was highly watersoluble, stable in water, blood and lymph, and impervious to sunlight. Furthermore, TGS121 was tested for anti-inflammatory properties *in vitro* and *in vivo* (Krajewska, 2021). The obtained results showed significant anti-inflammatory activity in colitis and gave a very solid basis for further preclinical investigations of this gold(III) complex.

Our distant goal is to examine the anticancer potential of new gold(III) derivatives. In this study – as an introduction to the consecutive investigation – we attempted to define the most probable molecular targets for the novel gold(III) complex TGS121. We propose that this compound may be a good candidate for further assessment of its safety and utility as a potential antitumor drug.

MATERIALS AND METHODS

SYNTHESIS OF TGS121

The chlorite-cyanide complex of monoionic gold (III) was prepared as described by Krajewska et al. *(*Krajewska, 2021). Briefly:

Pure metallic gold was dissolved in a mixture of concentrated hydrochloric and nitric acid in a molar ratio of 3:1 Next step was: heating with concentrated HCl, followed by removing liquid

acids from the gold (III) salt until the dry gold salt was obtained. The product was dissolved in aqua regia – in order to obtain clusters of gold (III) chloride smaller than 11-atom. Then, small gold clusters were treated once again with 6M HCl – to obtain an orange-red salt of gold (III) chloride, the analysis of which showed the presence of practically pure Au_2Cl_6. The product was treated with NaCl in a presence of water that lead to formation of a compound with the formula $Na_2Au_2Cl_8$. Next, the salt was treated with 6N HCl to obtain a solution of $HAuCl_2*H_2O$ monatomic gold salt with a pH of approximately 1.0. In the next step, the solution was neutralized by NaOH to pH 4-5 followed by the addition of $NaClO_2$ until a stable complex of gold (III) with chlorine dioxide and sodium chloride was obtained, with the formula: $NaAuCl_4* ClO_2*(NaCl)z$, where z is a number over 30.

The last step after neutralization with NaOH to pH 7.8, comprised of treatment with alcoholic sodium cyanide solution until end product was obtained. Recently, well-soluble in water complexes of mono-ionic gold (III) were neutralized to pH 7.4 with 0.1 M NaOH. Next, redistilled water was added to the volume of 1 dm^3.

The subject of synthesis was termed TGS121 and was diluted tenfold with saline (9 g/dm^3 NaCl) The chemical formula of TGS121: $[Au(CN)_4]_2*(ClO_2)Na$ and a diagram illustrating the composition of the molecule is shown in figure 1.

CELL CULTURE

For experiments we used parental normal fibroblasts NIH3T3 (ATCC) and their variant with Ha-Ras mutation – named Ras-3T3. The NIH3T3 cell line is a nonmalignant murine fibroblast cell line derived from NIH Swiss mouse embryo culture. The Ras-3T3 cell line was provided by Dr. H. Maruta (Ludwig Institute for Cancer Research, Victoria, Australia). The Ras-3T3 cells were obtained as described previously (Maruta, 1991). Briefly, normal NIH-3T3 fibroblasts were transfected with the v-Ha-ras oncogene inserted into the mammalian retroviral vector pMV7, leading to tumorigenic Ras-3T3 cell line.

Fibroblast cell lines were cultured in Dulbeco (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) (all from Sigma-Aldrich). Cells were kept in 25 cm^2 tissue flasks (Greiner, Berlin, Germany) in a humidified atmosphere containing 5% CO_2 and passaged every 2-3 days.

VIABILITY ASSAYS

Cells were treated for 24 or 48 h with increasing concentrations of TGS121. Relative cell viability was achieved by means of PrestoBlue Assay (Promega Corporation Madison WI) according to manufacturer's instructions. Absorbance of the experimental solution was measured directly in plates using OmegaStar Fluorescence reader at 550 nM.

The cytotoxic/cytostatic effects of novel compounds on culture cells were examined *in vitro* using PrestoBlue assay (Invitrogen, Carlsbad, CA), as previously described (Strus, 2021). Briefly, cells (5 x 10^3 cells/well) were seeded in 96-well microtiter plates (BD, Biosciences, San Jose, California, USA) and incubated with serial dilutions of TGS121. TGS121 was added in quadruplicate to a final volume of 200 μL. Appropriate volumes of culture medium were added as controls. After an incubation period of 24 or 48 hours, cells were stained with 25 μl PrestoBlue ready solution for 20 min, according to manufacturer's instructions. Fluorescence of experimental solution was measured directly in plates using OmegaStar Fluorescence reader (BMG LABTECH, Ortenberg, Germany) at 560/590 nm excitation/emission, respectively. Cytostatic/cytotoxic effect was expressed as relative viability of treated cells (% of control cells incubated with medium only) and was calculated as follows: relative viability = (Ae - Ab) x 100/(Ac - Ab), where Ab is background absorbance, Ae is experimental absorbance and Ac is the absorbance of untreated controls.

RESULTS

THE STRUCTURE OF NOVEL GOLD COMPLEX TGS121 AND ITS POSSIBLE INTERACTIONS

The obtained gold(III) complex with the formula $[Au(CN)_4]_2$ $(ClO_2)Na$ is a sodium salt of chloride dioxide associated with gold(III)-cyanide group complex (fig. 1). Particular chemical groups and molecules comprising the TGS121 complex ale displayed in figure 1. The figure also contains marking of the most evident relations between com-pound's composition and

possible chemical interactions with cellular targets. Relatively low molecular mass (692.5 g/mol) and the form of sodium salt make this compound well water soluble and stable in physiological fluids like a serum. Moreover, such a molecule can bind to albumins and in this way it can be also transported in living organisms. Chloride dioxide is a free radical containing one electron at incompletely-filled antibonding orbital. The presence of such unpaired electron makes this free radical actively interacting with various biologically active molecules, especially with thioredoxin complex. Eight cyanide groups can interact with thioredoxin, as well as with oxygene chain (complex). The presence of two Au molecules can result in interaction with DNA and with active places of some kinases, like MEK/ERK, PKC.

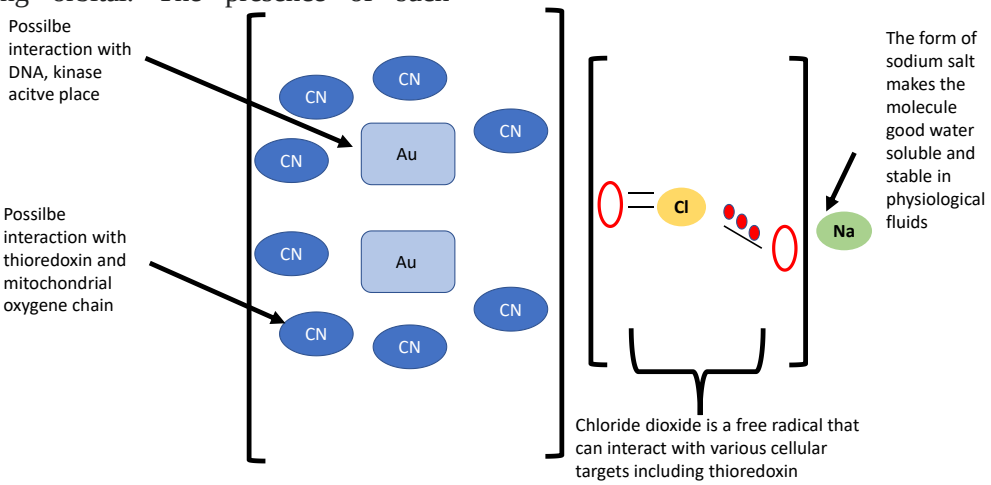

Figure 1. Molecular elements comprising the novel TGS121 complex. Arrows indicate elements possibly interacting with molecular targets in cells

TGS121 SELECTIVELY INDUCES DEATH OF RAS-3T3 TRANSFECTED FIBROBLASTS

The perfect anticancer drug should kill malignant cells selectively and leave non-tumor cells without damage. To check if TGS121 represents that case, we examined parental normal fibroblasts and its malignant variant with Ha-Ras oncogene hyperactivated. In Ras-3T3 neoplastic cells, the compound TGS121 induced concentration- and time-dependent effect with IC50 at 87.5 ug/L and 160 ug/L, for 24h and 48h, respectively. For non-malignant NIH3T3 cells, respective IC50 values were considerably higher (tab. 1).

Table 1. Inhibitory Concentrations of 50% (IC50) for TGS121 in malignant and non-malignant mouse fibroblasts indicate that higher TGS121 concentrations are needed for non-malignant NIH3T3 cells to inhibit cell growth of 50%

	Cell line	Time of incubation (h)	
		24	48
IC50 (µg/L) for gold(III) complex TGS121	Ras-3T3	87.5	160
	NIH3T3	350	3500

Consequently, we calculated the selectivity index for both incubation times. Our calculations showed that the novel gold complex is selective for Ha-Ras transfected malignant cells (tab. 2).

Table 2. The tumor-selectivity index (TS) was calculated by the following equation: TS = mean IC50 in normal cells/mean IC50 in malignant cells

	Compound	Time of incubation (h)	
		24	48
Selectivity index	TGS121	4	21.9

DISCUSSION

POSSIBLE APPLICATIONS OF TGS121

We obtained novel gold(III) complex TGS121 and studied its antitumor activity in Ha-Ras transfected fibroblasts in comparison to the parental non-malignant NIH-3T3 fibroblasts. The novel TGS121 compound turned out to be active against malignant cells at low doses (ng/mL), its effect was time- and dose-dependent. Low effective doses of the drug may mean low toxicity to healthy cells. TGS121 was not only active for Ha-Ras transfected malignant cells, but also was less toxic for non-malignant fibroblasts. The selectivity index was 21 at 48 h (tab. 2) indicating twenty times less toxicity for normal cells. It may be surprising that the selectivity index after 24 hours is only 4. But this short-time effect on normal cells may be only cytostatic effect. Normal cells may overcome cell death by transient cell cycle arrest, that is impossible for unhampered dividing malignant cells that are destinated for death due to accumulation of errors. Such protective effect was recently shown for podophyllotoxin complex in context of normal human keratinocytes (Strus 2021).

Because deregulated RAS/RAF/MEK pathway drives uncontrolled divisions of tumor cells, drugs selectively targeting cells with Ras hyperactivation can be widely used in cancer treatment (Khojasteh, 2021; Sugiura, 2021; Feleszko, 2000).

In particular three isoforms of Ras protein are recognized: Ha-Ras, N-Ras, Ki-Ras. The protein sequence is 80% identical between them, however, oncogenic mutations of the different isoforms predominate in different tumors. For example, Ha-Ras mutations are found in carcinomas of the bladder, kidney, and thyroid; N-Ras mutations are found in myeloid and lymphoid disorders, liver carcinoma, and melanoma; whereas Ki-Ras mutations predominate in colon and pancreatic carcinoma (Zhang, 1997).

Such oncogenic Ras mutations have been found in about 40% of human cancers and are thought to be a critical factor in the proliferation of these tumors. Thus, TGS121 is promising drug candidate for cancer treatment. Moreover, its physicopharmacological properties also confirm the possibility of using it directly as a drug. Notably, TGS121 complex is water soluble, its solution is stable in neutral pH, is stable at room temperature, the solution is clear and don't form any debris even if stored for several months, we can assume this compound can be given systemically in a form of intravenous infusion. For proper indication of novel TGS121 more studies are needed, extended cell studies, followed by preclinical and clinical ones. Especially there is needed analysis of TGS121 activity in human tumors with known Ha-Ras mutations (like bladder, kidney and thyroid cancer), but also in malignancies with other Ras mutations, since all Ras isoforms share 80% of identity.

POSSIBLE MECHANISMS OF ACTION OF TGS121 IN COMPARISON TO OTHER GOLD(III) COMPLEXES

DIRECT DNA DAMAGE

The model anticancer metallodrug is represented by various platinum complexes, like cisplatin or oxaliplatin, which target DNA (Dasari, 2014). Due to the isoelectronicity and isostructurality of gold(III) compounds to cisplatin, many scientists proposed DNA as the first target of these metallodrugs (Crooke, 1981). Hadjiliadis et al. *(*Hadjiliadis, 1981) proved that $HAuCl_4$, a gold(III) complex, can react with nucleosides. Another study showed that a number of gold(III) complexes may interact with DNA, although by a different chemical mechanism than cisplatin, while some were shown to exhibit an analogous to cisplatin mode of action, but significantly weaker (Mirabelli, 1986). In the same study, investigators established that coordination ligands are a defining factor of gold-DNA reactivity and it was revealed that only gold complexes containing halogen or pseudohalogen ligands interact with DNA. Based on these results researchers suggested increased lability of the gold-halogen to gold-carbon bonds (Mirabelli, 1986).

DNA binding affinity studies performed on several gold(III) porphyrins using purified calf-thymus DNA indicated that the DNA and gold(III) porphyrin interaction appeared to be noncovalent and reversible (Kang, 2005). Wang et al. (Wang, 2007) determined the nature of the reaction between gold(III) porphyrin 1a and DNA. Their results showed that the investigated agent causes fragmentation of DNA *in vivo*,

rather than a connection of two purine bases on the same strand of DNA, like cisplatin. In case of gold porphyrins, the biological activity strongly depended on the nature of the ligand. The porphyrin ligand significantly reduced the redox reactivity and stabilized the gold(III) center (Che, 2003).

A cisplatin-like, DNA cross-linking mechanism was observed in gold(III) dithiocarbamate derivatives (Ronconi, 2006). Importantly, the cytotoxicity of the investigated potential drugs was higher than cisplatin, even if the long term stability of gold(III) – DNA adducts seemed to be low. Moreover, DNA cross-links were repaired less efficiently compared to those induced by cisplatin. According to the obtained results, despite some similarities, the mechanism of action is different from that of cisplatin.

For other gold(III) complexes, weak and reversible interactions with calfthymus DNA were observed in mononuclear bipyridyl gold(III) complexes (Marcon, 2002), polyamine complexes (Messori, 2001), and dinuclear oxo gold(III) complexes bearing bipyridyl ligands (Casini, 2006), whereas tight bonds to DNA were reported in chloroglycyl-histidine gold(III) compounds (Carotti, 2000).

The novel TGS121 compound – due to the presence of a gold(III) atom that is surrounded by small cyano groups can fit into the DNA groove and associate with DNA, with subsequent DNA damage by a free electron donated by the chloride dioxide group (fig. 1).

THIOREDOXIN REDUCTASE (TRXR)

Multiple cellular processes involve redox-sensitive signaling factors. The thioredoxin system is an essential component in many redox-regulated pathways. It consists of thioredoxin (Trx) and thioredoxin reductase (TrxR). Both proteins have two isoforms, namely cytosolic (Trx1; TrxR1) and mitochondrial (Trx2; TrxR2) (Holmgren, 1985). Thioredoxin reductase is a selenoprotein with a selenocysteine-containing active site (–Gly–Cys–Sec–Gly). Its main function is to maintain Trx in the reduced state and allow donation of electrons to disulfides in proteins (Arnér, 1989). Among Trx substrates, ribonucle-otide reductase (RR)(Holmgren, 1989), NF-kB, AP-1, p53 (Qin, 1995; Abate, 1990; Ueno, 1999), glucocorticoid receptor (Grippo, 1983), ASK1 (Saitoh, 1998), protein kinases C (Watson, 1999) and tumor suppressor PTEN (Meuillet, 2004) have been distinguished.

Gold(III) compounds are known to strongly and selectively target thiol, imidazole and selenol groups of proteins (Casini, 2008). TrxR has a selenol group within the active site, thus it was considered as a good target for gold(III) derivatives. This view is supported by Gromer et al. (Gromer, 1998) who showed that glutathione reductase, an enzyme structurally and functionally similar to TrxR, but devoid of selenium, is significantly less sensitive to inhibition by gold complexes (Gromer, 1998). An increased level of Trx and TrxR has been observed in a number of human tumors, including colorectal cancer (Raffel, 2003). The same authors showed a positive correlation between ele-vated Trx and TrxR levels and increased cell proliferation, implying that the thiore-doxin system may play a crucial role in tumor progression (Grogan, 2000). Therefore, TrxR could be considered as a possible target for gold(III) complexes, similarly to auranofin and some auranofin-like gold(I) compounds (Rigobello, 2004; Marzano, 2007).

Coronnello et al. described a series of organo-gold(III) compounds with excellent anti-proliferative properties on the A2780 ovarian carcinoma cell line. Notably, the examined agents selectively inhibited TrxR. The observed proapoptotic potential of organo-gold(III) compounds most likely resulted from direct interference with mitochondrial functions.

In search of the properties of gold(III)-based TrxR inhibitors, Engman et al. (Engman, 2006) evaluated the effect of the number of carbon-gold bonds in these complexes on their toxicity. Among complexes with none, one, two or three such bonds, complexes with up to two carbon-gold bonds were the most potent TrxR inhibitors. The inhibitory concentration of the studied compounds was insufficient to kill cells.

Summarizing, the optimal structure of gold(III) compound, as an inhibitor of TrxR, consists of two carbon-gold bonds and one exchangeable group that could interact with ligands (Engman, 2006).

Similarly, the novel gold(III) complex TGS121 contains eight cyano groups, which can form at least two carbon-gold bonds; the other cyano groups interact with thioredoxins as ligands.

PROTEIN KINASE C (PKC)

Protein kinase C (PKC) is a protein kinase involved in cellular proliferation, cell cycle control, differentiation, migration, and survival. Aberrant PKC expression, activity or localization has been observed in various malignant processes. Thus, inhibition of PKC can be a potential therapeutic strategy in cancer treatment. The PKC family, which consists of 15 isoforms, is subdivided into three groups: classical (conventional), novel and atypical. The difference between particular isoforms of PKC depends on their secondary messenger requirements. Therapeutic targets have been developed for several PKC isoenzymes and some have been examined in clinical trials. For instance, the atypical protein kinase C iota (PKCi), unlike other PKCs, does not depend on calcium, diacylglycerol and phospholipid, but may be regulated by 3-phosphoinositides or through other specific protein-protein interactions. PKCi was found to be targeted by some gold derivatives and two compounds, aurothioglucose and aurothiomalate, were shown to inhibit its activation. These compounds were found to inhibit the proliferation of cancer cells, including non-small cell lung carcinoma (NSCLC) (Nobili, 1986).

The formation of gold – PKC kinase adducts is also possible for the TGS121 complex. Moreover, the gold atom in the TGS121 can specifically target the active site of PKC or the free electron of the chloride dioxide moiety can affect signal transduction to downstream elements.

MAPK – ERK PATHWAY

The extracellular signal-regulated kinase (ERK1/2) pathway is an important signaling component of the cell. This protein cascade regulates many cellular processes, including proliferation and differentiation. Stably activated Ras/Raf/MEK/ERK pathway is responsible for progression in most human cancers. Surprisingly, there also exist data suggesting that ERK plays a crucial role in the regulation of apoptosis. The transient ERK activation stimulates cell proliferation, while longterm ERK activation rather induces apoptosis. Thus, prolonged ERK activation may induce cell death through the intrinsic or extrinsic apoptotic pathway. The proposed mitochondrial-dependent mechanisms include the up-regulation of Bax and p53, as well as the suppression of survival signaling associated with Akt (Zhuang, 2006). The main differentiating factor in this signaling pathway, producing either cell proliferation or programmed cell death, is the duration of ERK activation. Prolonged activation of ERK can lead to programmed cell death through FADD-independent caspase 8 activation (Cagnol, 2006).

Yamagishi et al. confirmed that ERK plays a crucial role in the execution phase of apoptosis. Moreover, they described the possible mechanism of ERK activation by the ASK1-p38 MAPK pathway (Yamagishi, 2005). Hsieh and Papaconstantinou (Hsieh, 2006) suggested that the ASK1-p38 MAPK pathway is regulated through the level of reactive oxygen species (ROS). Reduced thioredoxin has the ability to bind ASK1 and inhibit the function of this protein. However, after Trx oxidation, ASK1 is released in the active form. When the level of ROS is increased, ASK1 is not inhibited by Trx and can activate p38 MAPK, which leads to apoptosis.

Therefore, there is increasing research regarding the influence of gold-based agents on the ERK pathway. Some gold(III) complexes were shown to activate ERK in a pro-longed manner, as long as cells were incubated with the examined gold complex. This mechanism was found for gold(III)-dithiocarbamato complexes, which have been shown to increase the level of phosphorylated ERK1/2 in HeLa cells (Saggioro, 2007). Results of this study suggested that the investigated complexes can inhibit TrxR, which leads to an increased concentration of oxidized Trx. A consequence of this phenomenon is accumulation of hydrogen peroxide. As mentioned in the paragraph above, the lack of reduced Trx does not inhibit the ASK–p38MAPK–ERK1/2 cascade, which promotes apoptosis (fig. 2). Also, hydrogen peroxide accumulation has been shown to cause ERK1/2 phosphorylation, thereby enhancing the apoptotic effect (McCubrey, 2006).

There is also an opposite hypothesis about ERK involvement in apoptosis that is induced by gold complexes. Park et al. *(*Park, 2006) reported that gold(I) compounds lead to apoptosis by activation of p38MAPK, whereas activation of ERK is independent of the concentration of the agent. Moreover, gold(III) porphyrin 1a was shown to

cause cell cycle arrest at the G2-M and G0-G1 phases, as well as increase accumulation of p53 (Wang, 2007). Subsequent results proved that phosphorylation of p38MAPK, induced by the gold compound, was involved in the cell death process (Wang, 2008).

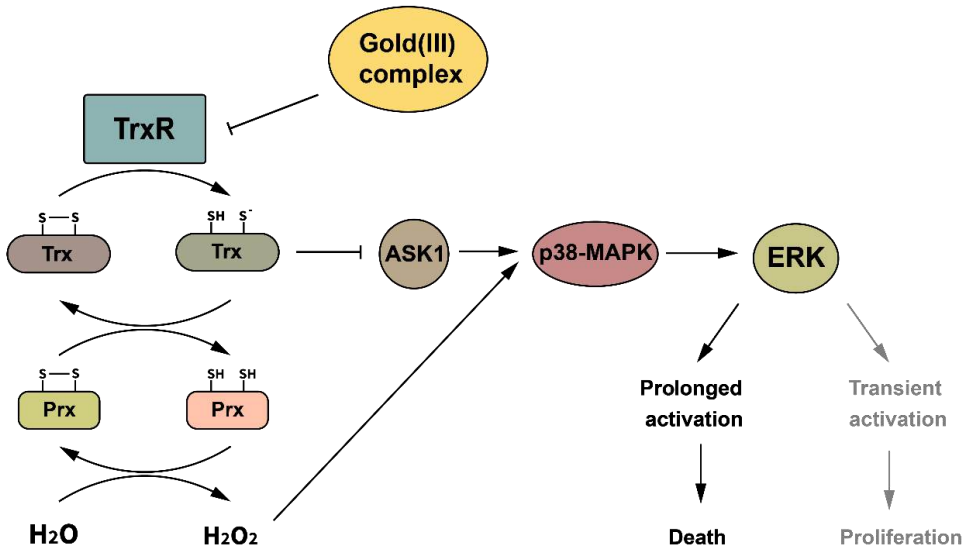

Figure 2. A proposed model for the role of ERK in apoptosis induced by the gold(III) complex

PROTEASOME

As the ubiquitin–proteasome pathway is essential for cell cycle regulation, apoptosis, angiogenesis and differentiation, it has recently been investigated as an intracellular target for gold(III) compound–induced cytotoxicity. Some gold complexes like the gold(III) dithiocarbamate compound were shown to inhibit proteasome activity and induce accumulation of poly-ubiquitin complexes, both *in vitro* in tumor cell lines, as well as in xenografts resected from experimental animals (Arsenijević, 2012).

These data suggest that proteasomes may be a target for gold(III) complexes and confirm that inhibition of the proteasomal activity could be one of the mechanisms of action of these compounds, including TGS121.

CONCLUSIONS

The novel compound TGS121 can exert multimodal effects in cells. These effects arise from the structure of TGS121, as it is a water soluble, relatively small molecule, containing various active groups. Thus, this molecule has the potential to become an anticancer drug, in particular since it is selective for Ha-Ras-transformed malignant cells in comparison to parental non-malignant cells.

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The role of liquid biopsy in lung cancer – from detection to disease control

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ABSTRACT

Lung cancer is one of the dominant causes of all cancer deaths. In 2020 worldwide there were 2.2 million patients diagnosed with lung cancer, and 1.8 million patients died at the same time. Based on the data from the European Cancer Information System, 318,327 patients were diagnosed with lung cancer in Europe in 2020.

Lung cancer symptoms, e.g. persistent cough and shortness of breath often fail to be recognised properly, which delays a visit to a specialist. Finally, most symptoms are diagnosed in stage IV of the disease, where therapeutic options are rather limited. Surely, there is an urgent clinical need in this area waiting to be recognised. Scientists all over the world are trying to find various solutions to better detect, predict forecast and avoid relapse of disease using novel techniques called "liquid biopsy". Circulating tumour DNA (ctDNA) is a biomarker that holds promising potential. One of the fractions of all Circulating Cell Free DNA (cfDNA), circulating tumour DNA is degraded and fragments of the DNA are released passively from apoptotic and necrotic cancer cells. Moreover, it has been discovered that cancer cells can also actively release ctDNA owing to the mechanism of producing extracellular vesicles (EVs).

The biology and the methods of the ctDNA identification will be presented in this narrative review. Further-more, it will be focused on presenting the role of ctDNA in screening and early diagnosis as well as on the effect of different concentrations of ctDNA on prognosis, staging, patients' stratification and detection of MRD (Minimal Residual Disease) for the group of lung cancer patients. It will be also highlighted that the ctDNA can be considered biomarker in terms of immunotherapy usage in different subgroups of patients. At the end, the review will focus on the future directions with innovative approaches that can potentially improve detection, therapy and finally make it possible to accurately predict and monitor the disease.

INTRODUCTION

CHARACTERISTICS OF LUNG CANCER

Based on the newest estimations provided by GLOBOCAN 2020, there were 19.3 million patients with a diagnosis of cancer, nearly 10 million of whom died. Lung cancer was responsible for 2.2 million cases and 1.8 million deaths. It is worth mentioning that lung cancer is now the second most frequent diagnosed cancer with the highest mortality in men, however, in women it is third most common cancer preceded only by colorectal and breast cancers (Sung, 2021). In Europe, lung cancer is the second most common cancer in males, preceded only by prostate cancer. As far as females are concerned, the incidence in Europe is exactly the same as globally. In 2020, there were about 320,000 patients diagnosed with lung cancer in the EU with over 257,000 deaths accordingly (OECD, 2020).

It is common knowledge that main risk factors for developing lung cancer remain unchanged and include tobacco smoking and environmental factors such as: air pollution, arsenic in drinking water, and exposure to asbestos. Increasing cases of the disease can be broadly prevented by implementing tobacco control policies and regulations (Carioli, 2021). Unfortunately, survival rates of patients diagnosed with lung cancer after 5 years are extremely low ranging from 10% to 20%. Survival rates are slightly higher in Japan (33%), Israel (27%) and the Republic of Korea (25%) (Allemani, 2018).

Lung cancer can be divided into two main groups: Non-Small Cell Lung Cancer (≈85% cases) and Small Cell Lung Cancer (≈15 % cases). The former (NSCLC) has different origins and is being categorized into three subtypes: Adenocarcinoma (LUAD, alveolar type II epithelial cell), Squamous Cell Carcinoma (LUSC, basal epithelial cell) and Large Cell Carcinoma (LCC); various epithelial cells. The later (SCLC) has its origin in neuroendocrine cell linage (Sánchez-Ortega, 2021).

It is important to note that survival rates depend on several factors, including the stage of the disease, histology type, and genetic alterations. In general, the prognosis for lung cancer patients remain poor. The 5-year survival rate for NSCLC patients is very low (about 16% at 5 years). In case of SCLC patients, the 5-year survival rate for people with SCLC is currently

7% (Sánchez-Ortega, 2021). It has been proved in multiple international randomized clinical trials that the introduction of annual low-dose computed tomography may significantly improve early diagnosis and reduce lung cancer mortality (Aberle, 2019).

Currently, we are witnessing a rapid development in precision oncology. These achievements greatly improve patients' outcomes, however, as indicated above there is a lot of space for improvement. Nowadays, we can describe a few examples of various genetic NSCLC (adenocarcinomas) growth drivers, which have contributed to developements in targeted therapies and immunotherapies. Currently, patients suffering from advanced lung cancer other than squamous cell carcinoma (SCC) should undergo genetic tests in clinical practice for the presence of EGFR, ALK and ROS1. The presence of these mutations is an important predictive factor for targeted therapies in terms of using inhibitors of EGFR (e.g. afatinib, erlotinib, gefitnib, osimertinib) and ALK or ROS1 inhibitors (e.g. crizotinib) (Lindeman, 2018). It was established that lung cancer, along with other cancer types, demonstrates a high genomic instability with the progressive acquisition of genetic alterations considered as: point mutations, epigenetic alterations etc. resulting in an expansion of complex diverse disease (Di Capua, 2021). Therefore, future directions will be focused on the development of various targeted therapies along with the evolution of standardized techniques of detecting biomarkers, which proves helpful in the disease control. Detection of circulating tumour DNA (ctDNA) has such a potential due to the fact that serum biomarkers are not generally used and surveillance is only based on clinical and radiological examinations (Schneider, 2020). Tumour originated DNA is released into various body fluids from: the primary tumour, metastatic lesions, circulating tumour cells (CTCs), minimal residual disease (MRD) (Li, 2020).

PURPOSE OF THE REVIEW

The main purpose of this review, based on the available literature, is an update and revision of current knowledge on the subject of the usage of detecting ctDNA in lung cancer patients. Detecting ctDNA, especially in case of non-small cell lung cancer (NSCLC), holds a big potential in early screening and disease control such as: detection of MRD, and further impact of next therapeutic options.

SEARCH STRATEGY AND SELECTION CRITERIA

To research the subject, the PubMed/MEDLINE, European Cancer Organization, Science Direct, Scopus databases were searched in a non-systematic manner for relevant publications. Articles that were published until August 2021 were included. Retrieved articles were filtered to remove duplicates and irrelevant results. The reference lists of the selected articles were checked for any other publications pertinent to this manuscript. The following key words were used: ("lung cancer" OR "non-small cell lung carcinoma") and ("ctDNA" OR "cfDNA" OR "liquid biopsy"). The research was limited to the available English reports (abstracts or full texts). All reports were selected (original and review articles) focusing on the ctDNA in the context of lung cancer diagnosis and therapy.

REVIEW

BIOLOGY OF "CFDNA – CTDNA" AND THE TECHNOLOGY OF DETECTION

Looking back on a timeframe, the first records of detection of cell free DNA (cfDNA) was date back to 1948, to the work of Mandel and co-researchers (Mandel, 1948). In the early 80's (1983) Shapiro with the team proved that there is a clear correlation between malignant and benign tissue and corresponding cfDNA concentrations (Shapiro, 1983). Cell-free DNA is released mostly from cells through mechanisms of apoptosis, necrosis, and also through active secretion in the process of forming extracellular vesicles (EVs). Apart from blood draw, the cfDNA can be detected in various body fluids such as: cerebrospinal fluid, urine, saliva and pleura (Wan, 2017). Elevated levels of cfDNA are described in various pathological conditions e.g. cancer, sepsis, autoimmune diseases and in particular, in physiological conditions such as pregnancy or intense physical exercise. As a result, the cfDNA level is studied as a potential biomarker for early diagnosis as well as for the diagnosis and prognosis (Khier, 2018). Unfortunately, there is insufficient evidence to support the pathophysiological role of cfDNA. Pre-

sumably, this is a byproduct of cellular processes/cellular stress (nonspecific biomarker of tissue damage). The role of cfDNA when released from healthy tissues is unclear.

Usually cfDNA are nucleosome protected 150-200 bp sized fragments, and have 2-hour half-life time (Perez-Barrios, 2016). However, in non-small cell lung cancer it has been determined that a molecule lasts for 35 minutes. (Chen, 2019). Different lengths of released fragments depend on the caspase endonuclease, which specifically splits DNA fragments. The newest studies have shown that the fragmentation pattern understood as length of released DNA is strictly dependent on tissue origin (Snyder, 2016). It important to highlight that ctDNA is one of the fractions of all cfDNA, no matter if it comes from healthy or cancerous tissue. The concentration of cfDNA in blood plasma is generally low ranging from 5-10 ng/mL, whereeas the fraction of ctDNA varies from 0.1% to 30% of the total cfDNA and is strictly dependent on the tumour size and disease stage (Crowley, 2013).

Taking into consideration low concentrations of cfDNA for liquid biopsy, samples require strictly defined conditions. Plasma is preferred over serum, due to a high risk of lymphoid cells lysis that lead to the deafening of ctDNA, finally giving false positive results. To avoid undesirable process of blood cells lysis, samples should be processed maximally within 4 hours from the collection in room temperature or within 24 hours at 4°C. To prolong the stability of the samples, there are also available commercially produced collection tubes (e.g. Roche Diagnostics, Qiagen). Those tubes contain leukocyte stabilizing agents that are capable of keeping the integrity of samples for at least 48 hours or even a week in a room temperature (Nikolaev, 2018).

Detection of ctDNA has been improved over the years, which has increased sensitivity and improved correlation with the results of tumour biopsy specimens. Detection of ctDNA requires the presence of mutations that can be detected by various sequencing techniques (Filipska, 2021). The biggest challenge in performing the analysis is to detect ctDNA in the background of cfDNA originating from healthy tissue, where allelic copies of mutated genes are pretty low (Crowley, 2013). In targeted methods of detection, better sensitivity scores can be observed, together with reduced scope in the genome (Ross, 2011). For example, the sensitivity of ddPCR technique varies from 74% to 82% with the specificity from 63 to 100% (Sacher, 2016). NGS techniques have even higher sensitivity and specificity, respectively ranging from 79% to 100% and 94-100% (Paweletz, 2016). Unfortunately, there is currently no "golden standard" in the detection of ctDNA. In general, the approach should fit the stage of the disease (fig. 1) (Riva, 2016). For example, PCR-based methods are quite sensitive and cost-effective, nevertheless, they also hold serious limitations. In the setting of NSCLC they are not recommended for detecting ALK and ROS rearrangements in ctDNA. Moreover, methods based on PCR are able to interrogate discreet and genetic mutations, and are limited in terms of the number of genetic targets that assays can detect (Rolfo, 2018).

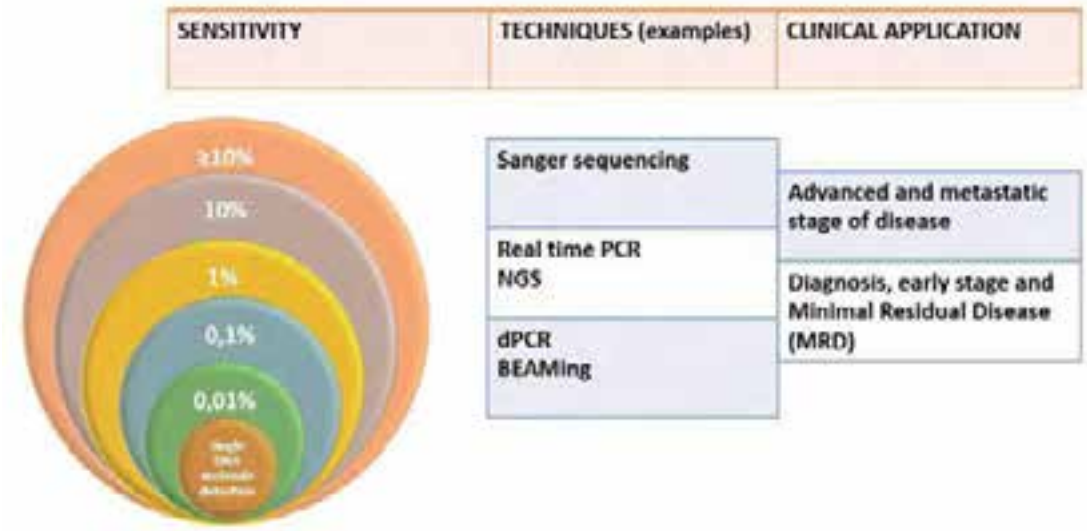

Figure 1. Different ctDNA detection techniques and their sensitivity versus clinical application (based on Riva, 2016)

Researches have already described a number of various molecular changes that cause lung cancer. Due to these findings patients' outcomes have significantly improved over the years, however, poor disease prognosis in late stages (III, IV) remain unchanged. The first symptoms of the disease, such as: shortness of breath, persistent cough, unintended weight loss, generalized weakness, are ignored by the patients (Gridelli, 2015). Thus, there is a reasonable unmet clinical need to develop noninvasive biomarkers such as ctDNA.

It has been proved that ctDNA genotyping can be a successful method for the early diagnosis of lung cancer (Li, 2020). It is important to highlight that a quick diagnosis allows early intervention and significantly improves survival rates (Cassim, 2019).

In trials conducted for various cancer types, there was approximately 82% sensitivity to a ctDNA detection in stage IV of the disease, however, in stage I of the disease, the sensitivity dopped to 47% (Bettegowda, 2014). Another trial involved female NSCLC patients who were tested for the presence of ctDNA. Plasma ctDNA, blood cell ctDNA, pleural effusion DNA samples where collected from these patients and the analysis justified that Next Generation Sequencing, droplet digital Polymerase Chain Reaction (ddPCR) was more sensitive and reliable than the usage of amplification refractory mutation system (ARMS) in terms of detecting ctDNA Epidermal Growth Factor Receptor (EGFR) mutations, specifically L858R and T790M mutations of early stage NSCLC. The detection of these alterations is important in terms of treatment with Tyrosine Kinase Inhibitors (TKI) (Rebuzzi, 2019). Moreover, in 2018 Cohen with the team developed CancerSEEK (Cohen, 2018). The test can detect eight different human cancer types by measuring concertation of circulating polypeptides and mutations in ctDNA. In case of lung cancer, detection rate reached 75%. It appears that the combination of detection levels of eight proteins and the presence of mutations in 1,933 distinct genomic positions can be considered to be an opportunity to diagnose lung cancer before the presence of radiological lesions, however, further studies on a bigger cohort of patients should be performed.

Advantages in the abovementioned techniques are undeniable, however, it is important to highlight the presence of the phenomenon called clonal hematopoiesis (CH). This condition, in some of cases, can be a source of false-positive results. It is considered that detectable mutations of cfDNA/ctDNA can lead to overdiagnosis. Clonal hematopoiesis (CH) is a typical age-related spread of white blood cells that carry somatic mutations, which is linked with an increased risk of hematological malignancies, cardiovascular diseases and other all-cause mortality (Silver, 2021). CH is caused by point mutations in genes associated with myeloid malignancies, chromosomal changes and loss of heterozygosity, whereby nonmalignant progenitor and hematopoietic cells acquire genetic alterations and may involve canonical CH genes. These genes include: tet methylocytosine dioxygenase 2 (TET2), DNA methyltransferase 3 alpha (DNMT3A), ASXL transcriptional regulator 1 (ASXL1), Janus kinase (JAK), tumourigenesis drivers, such as phosphatidylinositol 3-kinase (PI3KCA) and the abovementioned epidermal growth factor receptor (EGFR) (Liu, 2018; Hu,2019). The incidence of CH is ambiguous, but raises to 10-20% in individuals above the age of 70 (Silver, 2021).

The majority of cfDNA originates in hematopoietic cells, but those clonal hematopoiesis (CH) alterations in cfDNA are widely detected in serum and plasma. It means that with inappropriate controls, those mutations can be considered tumour-originated, causing misdiagnosis. High sensitivity analyses of cfDNA unveiled approximately 60-90% of CH mutations in patients without cancer, which proved that this condition is age-related (Liu, 2018). To ward off those false-positive results, there will be a need for introducing a procedure of white blood cells' control, that will include analyses of fragment length discrimination and deep error controlled sequencing (Filipska, 2021). Fortunately, this method is technically achievable and simple to conduct, although it doubles the costs and significantly reduces cost effectiveness (Chabon, 2020).

THE SIGNIFICANCE FOR PROGNOSIS PREDICTION AND TREATMENT CONTROL

As it has been described, a lung cancer patient has significantly higher total concentrations of cfDNA than healthy individuals. The total amount of cfDNA and ctDNA is correlated with tumour progression and the number of metastatic sites. Patients with different concentrations of ctDNA can be stratified into two groups: low-ctDNA concentration and high ctDNA concertation, which clearly guides the prognosis (Li, 2020). For example, the status of EGFR mutation significantly impacts patient outcomes. Alteration of EGFR gene leads to a dysregulation of cellular growth and proliferation. The frequency of EGFR mutations in NSCLC varies from 13% to 22%, depending on patient's ethnicity with a higher incidence in Asians (Antonoff, 2012). The status of EGFR ctDNA mutations has been checked in various clinical studies. One of them proved that the presence of EGFR ctDNA in patients with metastatic disease significantly reduces the time of progression free survival (PFS) and overall survival (OS) (Kim, 2019). There were several other clinical studies supporting the argument that detection of EGFR ctDNA shortens patients' outcomes measured as PFS and OS (Mok, 2015; Lee, 2016). Nonetheless, other studies showed contradictory correlation between the ctDNA EGFR presence and its impact on PFS, OS (Fan, 2017; Mao, 2015).

Fan et al. published metanalysis that reported PFS and OS stratified by the presence of EGFR and KRAS mutations in ctDNA in advanced NSCLC lung. The pooled analysis showed that EGFR mutations in ctDNA were significantly improving the PFS (HR = 0.64, 95% CI [0.51-0.81], I^2 = 0%, p < 0.001) in patients treated with EGFR-TKI. A trend was also observed of prolonged OS in terms of EGFR ctDNA presence. On the other hand, the presence of KRAS mutations in ctDNA was correlated with a prediction of worse PFS (HR = 1.83, 95% CI [1.40-2.40], p < 0.001) and OS (HR = 2.07, 95%CI [1.54-2.78], p < 0.001) in advanced NSCLC patients treated with chemotherapy. The abovementioned conflicting results may be due to the application of different inclusion/ exclusion criteria, as well as various generations of TKI (currently three generations are used in treatment) (Zhang, 2016), and finally due to small sample sizes.

Undoubtedly, globally performed clinical studies with improved stratification of patients in terms of ctDNA detection will facilitate proper direction. Presently, there are two assay kits approved for the EGFR testing in liquid biopsies, TheraScreen EGFR RGQ PCR Kit (Qiagen) and Cobas EGFR Mutation test v2 (Roche Diagnostics). Generally, researchers aim to develop finelytuned approach to guiding lung cancer patients that will be approved by regulatory authorities for usage in clinical practice.

MINIMAL RESIDUAL DISEASE (MRD)

ctDNA is also under investigation for usage as a marker for detecting minimal residual disease (MRD) in patients who have undergone therapy for NSCLC. The intent of applying this approach comes from disadvantages of standard imaging. Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) feature limited sensitivity to detecting micrometastases compared to overt metastases (Li, 2020). There was a demonstrable decrease in ctDNA in patients that have undergone resection of NSCLC (Guo, 2016). In one study, over 50% of patients showed detectable ctDNA in the post treatment period, while 72% of patients had detectable ctDNA prior to the relapse of the disease. Moreover, the detection of ctDNA anticipated a radiological relapse by a median of 5.3 months (Chadhuri, 2017). Another study evaluated the dynamics of ctDNA testing. The presence of ctDNA one day after the surgery was not impacting the outcomes for patients understood to have had relapse free survival (RFS) and OS. How-ever, the presence of ctDNA 3 and 30 days after surgery was associated with poor outcomes (Chen, 2019). Surely, the introduction of detecting ctDNA defined as moni-toring the presence of MRD into regular clinical practice e.g. performing "liquid biopsy" during follow-up visits can significantly improve patients' outcomes. Implementing targeted treatment before the occurrence of detectable metastases can unquestionably prolong life expectancy.

CTDNA – BIOMARKER FOR IMMUNOTHERAPY

The introduction of immunotherapy into treatments of different types of cancer has showed promising responses depending of the subgroup of patients. In terms of these results, there is a need for searching biomarkers which will better stratify the patients (Filipska, 2021). One of the

recently launched clinical trials can change current clinical practices. BESPOKE study (NCT04761783 – May 2021) will examine the impact of SIGNATERA™ (personalized, tumour informed 16-plex Next Generation Sequencing assay for the detection of ctDNA) on treatment decisions on tumour assessment (NSCLC, Melanoma, Colorectal Cancer) and timepoints after the initiation of immunotherapy. Furthermore, researchers detect Histone-Lysine N-Methyltransferase SETD2 mutation in fusion drived NSCLC, which is not detected in patients with BRAF, KRAS, EGFR or MET mutations. These findings are important because ALK, ROS1 fusion mutations showed no clinical response to the treatment with immune checkpoint inhibitors e.g. antibodies targeting anti-PD-1 (nivolumab and pembrolizumab) and anti-PD-L1 (atezolizumab). Moreover, the PD-1/PD-L1 expression level is correlated with EGFR, ALK, and the treatment of patients with TKIs. Several clinical studies have shown that the level of expression of PD-L1 protein is upregulated in NSCLC cell lines that express an EGFR and the EML4-ALK fusion protein that are responsible for the progression of tumour (Zhu, 2020).

Although the development antibodies for the treatment of cancer is indisputable, not all NSCLC respond to the treatment. There is a need to validate the feasibility of ctDNA detection in large cohort clinical trials to maximize the outcomes for patients treated with immunotherapies (Yang, 2021).

CONCLUSION

It is certain that ctDNA-based liquid biopsies may be a forceful tool for diagnosing cancer, monitoring the disease, making predictions and obviously changing the current procedures in managing the patients with different types of cancer (fig. 2). In authors' opinion these techniques will provide detailed molecular information and may reduce a need for performing high risk invasive procedures as well as the execution of pathological assessment.

However, introducing ctDNA testing for regular practice in each and every case faces several barriers. The biggest obstacle is the requirement for proper specificity and sensitivity. Moreover, there is a need for lowering the testing costs. This problem stems from very low concentrations of ctDNA, especially in early stage of the disease and is also strictly dependant on tumour biology. The number of patients enrolled for ctDNA research is much lower than the respective number in other clinical studies. Due to the aforementioned reasons clinical interpretations of given results are presently rather hobbled. It will be crucial to test composite gene panels with well indicated endpoints to prove their clinical utility.

Certainly, further studies will deliver more "real-world" data which should and can improve all stages of ctDNA analysis starting from the isolation and finishing on the interpretation of received results. A combination of standard of care approaches with the abovementioned novel techniques can significantly change lives of patients suffering from lung cancer and also other types of cancer.

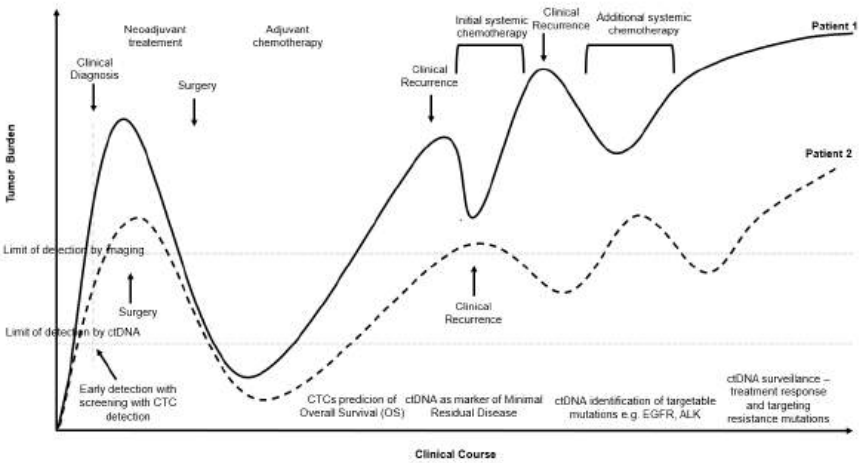

Figure 2. Graph indicating possible roles of ctDNA along with CTCs in the clinical course of patients suffering from Non-Small Cell Lung Cancer in comparison with standard of care (SoC) approaches. Patient 1 (solid line) demonstrates a standard "journey", but Patient 2 (dotted line) goes with the approach including the usage of ctDNA and CTCs in NSCLC treatment (based on Di Capua, 2021)

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Emperipolesis in neuroendocrine tumors of the thymus

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ABSTRACT

Emperipolesis is a biological phenomenon of rare origin and is characterized by a process in which a cell penetrates another living cell. In contrary to phagocytosis where the engulfed cell is killed or neutralized by lysosomal enzymes of the macrophage, in emperipolesis, the cell exists as a viable cell within another. Moreover, this cell can exit at any time without any structural or functional abnormalities for either of them. The process of emperipolesis is seen in many physiologic and pathophysiologic conditions. In this article we focus on the occurrence, pathogenesis and appearance of emperipolesis in the neuroendocrine tumors of the thymus. Moreover, we highlight the possible diagnostic and future therapeutic strategies in the treatment of thymic tumors.

INTRODUCTION

Neuroendocrine tumors of the thymus are classified according to World Health Organization (WHO) guidelines. Primary neuroendocrine tumors of the thymus (NETTs) are very uncommon and represent less than 5% of mediastinal and thymic neoplasms. They account for only 0,4% of all neuroendocrine tumors (Dinter, 2019). NETTs are classified according to WHO criteria into lowgrade typical carcinoids, intermediategrade atypical carcinoids (ACs), and two highgrade malignancies, large cell neuroendocrine carcinoma (LCNEC) and small cell carcinoma (SCC) (Dinter, 2019). To categorize tumors, morphology evaluation should be performed, with the assessment of parameters such as organoid nesting, rosette formation, peripheral palisading of tumor nests, and trabeculae (Dinter, 2019). This classification was made by determining the mitotic activity, cellular atypia and areas of necrosis (Moran, 2000). To classify a tumor as a typical carcinoid, it is identified to have no necrosis and a size of 0,5 cm or greater, AC is reported to have 2 to 10 mitoses per 2 mm with or without necrosis, whereas LCNEC and SCC have number of mitoses greater than 10 per 2 mm (Moran, 2000). According to this classification, AC and LCNEC are the most common subtypes in the thymus (Dinter, 2019).

The 3rd and 4th edition of the WHO Classification of thoracic tumors are considered to be most important. In accordance with the 2004 classification, WHO distinguishes A, AB, B1, B2 and B3 types of thymomas and thymic carcinomas and other seldom ones. (Marx, 2014; Petrini, 2014). The fourth edition is expanded to include an interdisciplinary perspective and improves histological and immunohistochemical diagnostic criteria in order to increase the diagnostic repeatability.

The nomenclature of the major thymoma types was retained in the 4th edition, as well as Masaoka-Koga system for the staging of thymomas (Marx, 2015). However, the term "combined NETTs" is no longer used, excluding type AB thymoma. Instead, there is a requirement to include all histologically diagnosed thymoma types, starting with the most important ones and quantified in 10% increments (Marx, 2015).

Primary neuroendocrine tumors of the thymus (NETTs), which include thymic neuroendocrine tumors, thymoma (TM) and thymic carcinoma (TC) are always considered to be malignant, and it is unrelated to subtype or histology of the tumor (Jeong, 2020; Marx, 2015).

In the past there was a problem with distinguishing between some thymoma subtypes and thymic carcinomas, because of morphological overlapping (Marx, 2014). Diffe-rences between thymomas and thymic carcinomas have been diagnosed by epigenetic and genetic methods and transcriptomic analyses, which showed different methylation patterns, expression profiles of antiapoptotic genes and specific mutations of epigenetic regulatory genes (Marx, 2015). Thus, interobserver reproducibility has been improved. Also point mutation in the GTF2I (general transcription factor 2-I) oncogene in all major thymoma subtypes and thymic carcinomas was observed, which indicates the common origin of the NETTs (Petrini, 2014).

The possible role of emperipolesis in neuroendocrine tumors of the thymus requires further clarification. In this review we discuss the previous findings in this area of expertise and the significance of this rare process, not much reported in the literature.

SEARCH STRATEGY AND SELECTION CRITERIA

The authors reviewed data published in 3 languages: English, German, and Polish between 1989 and 2020. Data were collected using keywords such as emperipolesis, entosis, and neuroendocrine tumors of the thymus. The following scientific databases such as PubMed, Google Scholar, Borgis, MEDLINE, and Cochrane Library were used to search for articles. The selected articles focused on determining the importance of emperipolesis in the pathogenesis, diagnosis and treatment of thymic neuroendocrine tumors. The number of articles selected was 54. In addition, this work was enriched with 7 manually selected materials that were related to the discussed topic. The strategy was aimed at presenting yet not entirely understood aspects of emperipolesis in the context of neuroendocrine tumors of the thymus as well as emperipolesis itself from various perspectives.

WHAT IS THE EMPERIPOLESIS?

Emperipolesis is characterized by the presence and movement of one cell within the cytoplasm of another. Emperipolesis is strictly related to cell-in-cell phenomenon, which can be associated with the prognosis of cancers (Wang, 2019). Histopathological screening shows an absorbed cell in a membrane-bound vacuole in the host cell. On occasion absorbed cells may continue to live for a short period of time after absorption. It is possible for an internalized cell to escape from the host cell, and it can survive after this process (Gupta, 2017).

The term "*emperipolesis*" originates from the Greek (*em* – inside; *peri* – around; *polemai* – wander about) and it was first reported and defined in 1950 as the active penetration of one cell by another (Humble, 1956). Wang and Li (2019) had discovered that emperipolesis can mediate natural killer cell-mediated tumor cell death, but requires membrane fluidity of the target cell, so that the interaction with natural killer cells could occur. The host tumor cell disintegration is preceded by lysosome-mediated degradation pathway after the emperipolesis (Xia, 2008). According to Overholtzer's report, natural killer cells sometimes can undergo mitosis inside the host tumor cell after emperipolesis and that indicates the further fate of heterogeneous cells in killer cell-tumor cell emperipolesis (Overholtzer, 2007).

Rosai-Dorfman disease (RDD) is a pathological condition in which emperipolesis occurs. It was first observed by Juan Rosai and Ronald Dorfman in 1969, and has been diagnosed by cervical lymphadenopathy, lymph node sinuses and emperipolesis that occurred within histiocytes (Rosai, 1969). In RDD a dense histiocytic infiltrate with emperipolesis is present. The infiltrate contains associated lymphocytes, plasma cells, and neutrophils (Cangelosi, 2011). However, emperipolesis is a diagnostic feature only when S100 protein is expressed in histiocytes (Juskevicius, 2001). Yet, due to variable morphology characteristics in xanthogranulomatous diseases, emperipolesis is the most important histologic feature in distinguishing it from RDD disease (Cangelosi, 2011).

CHARACTERISTICS OF THYMIC NEUROENDOCRINE TUMORS

Primary neuroendocrine tumors of the thymus (NETTs) belong to the group of tumors with high aggressiveness (the ability to form metastases in more than 80% of patients) and a relatively low incidence (Chaer, 2002; Filosso, 2017). NETTs account for only about 0,4% of all carcinoids and less than 5% of all the anterior mediastinal neoplasms (Yao, 2007; Filosso, 2017). Primary neuroendocrine tumors of the thymus are found predominantly in males, with a male to female ratio of 3:1 (Moran, 2000). They are most common in white males and are typically seen in the fourth or fifth decades of life, with an average age of onset of 58 years (Gaur, 2010). NETTs likely arise from Kulchitsky cells and localize primarily to the anterior mediastinum (Berman, 2020).

According to the WHO (2015), primary thymus neuroendocrine tumors are classified into two main histopathological types: well-differentiated (typical and atypical carcinoids) and poorly differentiated (small cell and large-cell neuroendocrine crayfish) (Travi, 2015).

Clinically, NETTs may manifest as follows: 1. asymptomatic, coincidentally detected on chest radiography for other reasons; 2. with symptoms due to displacement/compression/invasion of mediastinal structures; 3. associated with endocrinopathies; or 4. with symptoms due to

distant metastases, most commonly to the liver, brain, lung, or bone.

Primary neuroendocrine tumors of the thymus give many non-specific symptoms including chest pain, cough, dyspnea, superior vena cava syndrome, lingual nerve palsy, and diaphragmatic elevation due to damage to the phrenic nerve (Berman, 2020). In addition, half of the patients had lymph node involvement, but with no proven effect on reducing treatment efficacy (Filosso, 2017).

Approximately 50% of thymic neuroendocrine tumors are functionally active and have the ability to secrete hormones. Ectopic secretion of ACTH and serotonin can lead to paraneoplastic Cushing's syndrome and carcinoma, respectively. Less commonly, excessive secretion of somatoliberin (GHRH, growth hormone-releasing hormone) or growth hormone (GH) has been described, which can cause acromegaly (Melmed, 2009). People with hypertension, heart failure, diabetes, and arthropathies are primarily at risk of developing this condition (Bolanowski, 2014).

In addition, 25% of patients struggling with a thymic tumor have a coexistence of multiple endocrine neoplasia type 1 (MEN1) syndrome; one of the 8 contemporary multiple endocrine neoplasia syndromes (Gietka-Czernel, 2017). MEN1 is the leading cause of death among patients with thymic neuroendocrine tumors (Phan, 2010). Therefore, many authors recommend prophylactic thymic resection (parathyroidectomy) in patients with MEN1, which reduces the risk of cancer during life (Teh, 1998; Trump, 1996)

IMAGE OF EMPERIPOLESIS IN EPITHELIO-RETICULAR CELLS OF THYMIC TUMORS

Most thymic tumors have thymic epithelial cells that do not show cytological malignancy. Moreover, these cells are mixed with lymphocytes in different proportions (Verley, 1985; Lewis 1987). It appears, that the phenomenon of lymphatic emperipolesis, in which the intact cell is present in the cytoplasm of the larger cell, may occur in epithelioreticular cells. This issue however warrants further scientific evaluation. The subject of the thymoma in the context of emperipolesis is likewise not much reported in the literature.

In an ultrastructural study, Llombart-Bosch suggested that close contacts existed between the thymic lymphocytes and the epithelioreticular cells. This appearance was suggestive of emperipolesis (Llombart-Bosch, 1975). In another research conducted by Izard, the cytoplasmic structures resembled the embryonic epithelioreticular cells in the guinea pig thymus (Izard, 1966). Interestingly, Llombard-Bosch suggests that mitotic lympho-cytes are found throughout the tumor near E-R cells (epithelioreticular cells). Moreover, there is a morphological and lymphocytic death relationship, while the lymphocytes were in the cytoplasm of E-R cells. The onset of such necrosis is progressive nuclear pycnosis and secondary chromatolysis. By the time the cytoplasm was completely gone, the fatty degeneration and the mitochondrial vacuolization had started. The remaining monoliform reticular particles swallowed mesenchymal macrophages. Such cells were characterized by advanced degradation (Llombard-Bosch, 1975). Macrophages have the ability to phagocytose and to absorb what they phagocytize. They are classified as connective tissue and are associated with the body's defense mechanisms (Cichocki, 2002). In this case, the mesenchymal macrographs were randomized in the tumor stroma, but were more frequent near E-R cells. Moreover, phagocyte-ingested cell debris of lymphocytic origin were also present (Llombard-Bosch, 1975).

There are only very few publications on emperipolesis in the context of the thymus gland, and even less in relation to E-R cells. It seems that the topic of emperipolesis requires further attention and research. Similar observations to the two cases cited above were noted in epithelionreticular cell thymoma in carp. Lymphocytes were taken up by E-R cells. It therefore seems logical that there is some kind of cytoplasmic communication system between lymphocytes and E-R cells. Such a phenomenon can take place in the human thymus, as indicated by Golditeinand MacKay (1969) (Romano, 2004).

It is important to properly distinguish between thymoma and T lymphoblastic lymphoma using needle biopsy as this has serious consequences in further treatment. Among diagnostic criteria, a factor that favors thymoma is the demonstration of increased numbers of keratin-positive epithelial cells using immunohistochemical staining. Loss of keratin expression in neoplastic epithelial cells could lead to detrimental misdiagnoses (Adam, 2014). Notably, false-positive

or otherwise negative results of various tests may be related to the physiology of the cell itself, which may lose or gain certain properties under the influence of given factors or for unexplained reasons. Here the loss of keratin expression is observed. The research revealed that thymic epithelial tumors showed highly reduced expression of at least one keratin (Adam, 2014).

Moreover, emperipolesis in the form of thymocytes in the cytoplasm of epithelial cells was noticed in imprint cytology but was not noticed in a histological examination, which will be discussed in the next section (Nerurkar, 2000).

According to the research, emperipolesis was also noticed in an 83-year-old patient who underwent Chamberlain anterior mediastinotomy. The presumptive diagnosis was a thymic tumor versus lymphoma. It was suggested to consider the test sample as an atypical thymoma. Another suitable alternative might be a thymic carcinoma (Mackay, 1985).

Considering the aforementioned results, the image of emperipolesis in the thymus is rarely observed, and if it is noticed, it arouses curiosity. This phenomenon warrants further evaluation. The research on animals (guinea pig and carp) is aimed at high-lighting the importance of a holistic approach to the issue. Similar studies in animals can possibly be done faster, easier and in a larger population. Results may emerge sooner, and the similarities between the human thymus and animal glands, which already have been demonstrated.

EMPERIPOLESIS AS A KEY FEATURE IN IMPRINT SMEARS OF THE THYMUS

Among diagnostic imaging of the thymus, imprint cytology has not received much attention, because the organ is rarely sampled in routine surgical practice.

It appears that emperipolesis may not be noticed on histology, but, surprisingly in imprint cytology. Based on the presented research, a fragment of the thymus was mistakenly sampled as a pretracheal lymph node in order to exclude metastasis. Interestingly, the presence of thymocytes in the cytoplasm of thymic epithelial cells (emperipolesis) was the most significant feature in the imprints (Nerurkar, 2000). Imprint cytodiagnostic is useful, for example, in examining breast tumors. Contrary to histopathology, which is more time-consuming, imprint smear can take less than an hour. Moreover, imprint smear can do amastigotes that take a short course without the need for a pathologist (Sousa, 2014). In the study of Nerurkar, the emperipolesis was based on the ingress of thymocytes into the TNC. TNCs are thymic nurse cells, which are epithelial cells in the thymic cortex, nourish the thymus and can surround the thymocytes to form lymphoepithelial complexes. Importantly, the thymocytes in the cytoplasm in this case did not show signs of nuclear degeneration. So, for example, pyknosis did not occur (Nerurkar, 2000). Pyknosis is the process of a cell in apoptosis or necrosis and consists of irreversible chromatin condensation (Kroemer, 2009). Additionally, immunohistochemistry with keratin, which confirmed that thymocytes are double by TNC. The method also showed that thymocytes are alive but not proliferating. Such emperipolesis took place not only in the cortex, but also in the corticomedullary junctions (Nerurkar, 2000). Other scientists studying immunohistochemical characterization of nurse cells in normal human thymus had similar observations. Moreover, this study showed that internalized thymocytes retain their proliferative potential (Dispasquale, 1991). Imprint smear is a quick diagnostic method, e.g. for tumors, but the disadvantage is that it does not allow reliable results in the context of tumor infiltration (Mehar, 2014).

Among the available imaging techniques, observations with an electron microscope and phase contrast microscope are indispensable for distinguishing emperipolesis from phagocytosis (Shamoto, 1980). This can be more difficult to observe under a light microscope (Mackay, 1985).

Indisputably, a wide range of diagnostic methods is needed to fully diagnose and investigate a given tumor. Paradoxically, it appears that imprint cytology, being less advanced technique than fine needle aspiration (FNA) cytology or histology, enables demonstration of such rare phenomenon as emperipolesis. More studies are necessary for these findings to be placed in a proper perspective.

COMPARISON OF EMPERIPOLESIS AND ENTOSIS

Emperipolesis and entosis are very similar processes but differ in the pattern of action and the mechanisms involved. In the case of entosis, the predominant fate of internalized cells is

lysosomemediated degradation and non-apoptotic cell death (Peng Xia, 2008). Emperipolesis, on the other hand, is the process of entry and temporary 'storage' of one cell in the cytoplasm of another cell, but one that is histogenetically foreign. In emperipolesis, a cell exists as an intact living cell in the cytoplasm of another and can exit at any time without any structural or physiological abnormality for either (Amita K, 2011). Emperipolesis is thought to improve cell survival and help prevent cell apoptosis in the host cell. The engulfed cell can be destroyed and depending on its mode of death, there are different terms to describe this procedure. For example, non-apoptotic death can occur as a result of so-called "suicidal emperipolesis" (Benseler et al., 2011). Emperitosis (a combination of emperipolesis and apoptosis) can also occur. The host cell can also be destroyed; killing of lymphocyte-containing tumor cells has been observed (Wang et al., 2013).

Both emperipolesis and entosis require extracellular free calcium and adhesion molecules and an actin-based cytoskeleton (Peng Xia, 2008). To systematically define emperipolesis and entosis it is necessary to identify the key intercellular junction molecules involved in these processes.

Entosis – from the Greek "*entos*" – "within", involves the absorption of one cell by the vacuolar system of a neighboring cell of the same type, from the same population, due to the loss of linkages between the cell and the extracellular matrix. Nonapoptotic death of such a cell may then occur, in the absence of caspase-3, requiring autophagy by lysosomal enzymes, or it may divide and leave the parent cell by a transcytosis-like movement. It is suggested that entotic cell death should be defined as a new type IV cell death.

Entosis can occur under physiological as well as pathological conditions. As a result of entosis of tumor cells, the tumor cell may undergo:

- incomplete heterophagocytosis with removal of the remaining cancer cell outside the phagocyte or complete heterophagocytosis;
- pseudo-cannibalism – no change in the tumor cell;
- disintegration into glandular bodies, which remain in the cytoplasm of the cell;
- malignant transformation, i.e. benign tumor cell becoming malignant;
- progression of the malignant tumor cell;
- suppression of the tumor process by repeated uptake of the malignant tumor cells.

PATHOGENESIS OF ENTOSIS

Entosis is caused by cell detachment from the extracellular matrix and also enhanced by an imbalance in actomyosin contraction between neighboring cells. Entosis is mediated by E-cadherins and P-cadherins increasing cell adhesion in the absence of integrin signaling. The process also requires Rho GTPase, Rho kinase ROCK and myosin-based contractile force.

Moreover, entosis is favored by the presence of the Kras oncogene and the expression of epithelial cadherins E and P. Oncogenic transformation and mechanical deformability of the cell promotes the ability to engulf other cells, which usually leads to non-apoptotic death of such cells, but may also increase the metastatic potential of the tumor and induce changes in cell ploidy, leading to the formation of binucleated cells in culture (Gupta N., 2017).

Recent studies have shown that entosis can occur even when cells are attached to the matrix. It is presumed that mitosis is then the inducer of entosis. Also, it is thought that the lack of glucose in the growth medium may induce it by increasing the activity of AMP protein kinase (AMPK) (Xinlong Wang, 2019).

PATHOGENESIS OF EMPERIPOLESIS

Emperipolesis can be physiological, pathological or a pathognomonic feature of certain diseases. It is thought to be a form of temporary cell protection against carcinogens and chemotherapeutics, as this process is often seen in some mesenchymal tumors (multiple myeloma, acute and chronic leukaemia, myeloproliferation) and also during the use of cytostatic drugs. In pathological states it also occurs in Rosai Dorfman disease, which is a histiocytic proliferative disorder in which emperipolesis can be observed in lymph nodes with inflammatory infiltration and in cerebrospinal fluid. Emperipoietic erythroblast activity in the liver has been found to increase during periods of high

hepatic erythropoietic activity and relatively anemic fetal state.

Physiological findings include emperipolesis of erythroblasts by megakaryocytes in the fetal liver, emperipolesis of lymphocytes by human glial cells in the brain.

Free calcium molecules and adhesion molecules are important in emperipolesis, as well as the actin- and ezrin-based cytoskeleton (Xia, 2008). Emperipolesis has been shown to decrease by inhibiting actin polymerisation (Takeuchi, 2010). Abnormal P-selectin located in the demarcation membrane system of neutrophils and megakaryocytes has been proposed as a cause of emperipolesis in marrow fibrosis (Centurione, 2004). It is thought that also a lymphocyte function-related antigen-1 (LFA-1 or CD11a/CD18) that can mediate intercellular interactions between leukocytes and non-blood cells together with its ligand, intercellular adhesion molecules 1 (ICAM- 1/CD54), may be associated with emperipolesis (Reina and Espel, 2017).

Emperipolesis and entosis are two different phenomena. The process of emperipolesis occurs with the involvement of Ezrin, LFA-1 and ICAM-1. The engulfed cell can escape from the host or be killed. The host cell can be destroyed by the engulfed cell. In contrast, entosis is homotypic, in which E-cadherins and P-cadherins, the Rho-ROCK-actin/myosin pathway and actomyosin contraction imbalance play important roles. The absorbed cell may be killed or survive (Xinlong Wang, 2019).

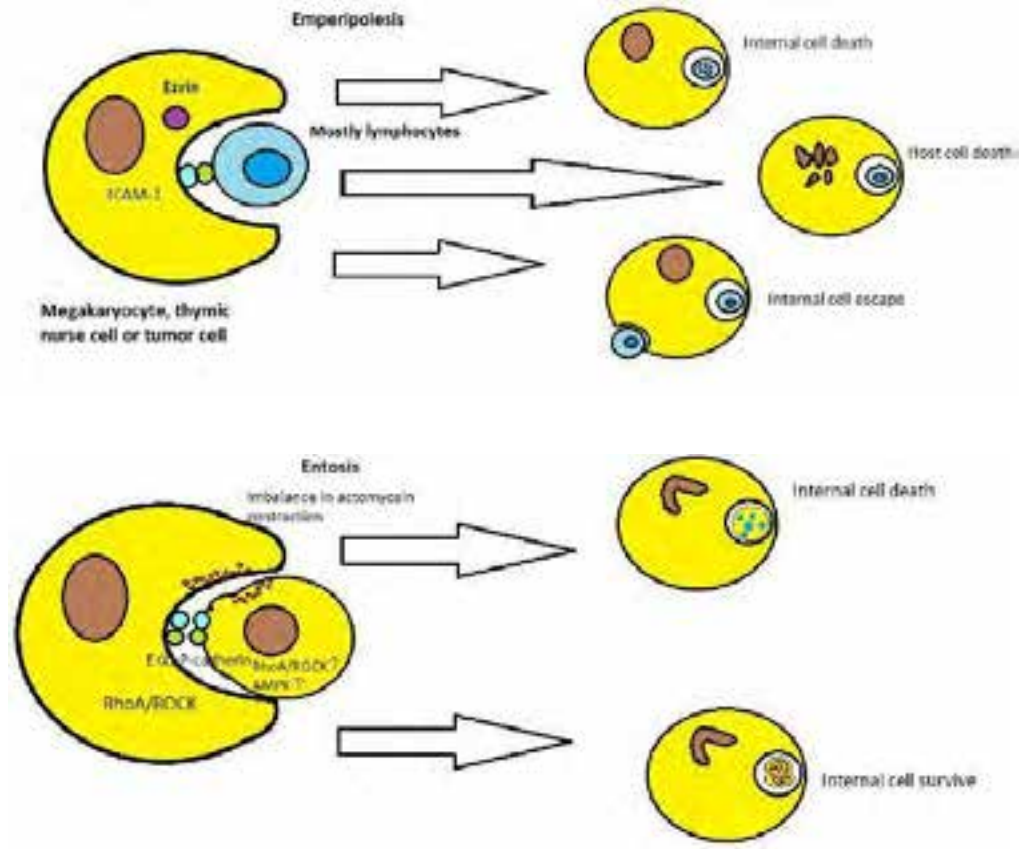

Figure 1. Emperipolesis and entosis – modified based on Wang et al. 2019

DIAGNOSIS OF NEUROENDOCRINE TUMORS

The standard procedure for the diagnosis of primary neuroendocrine tumors of the thymus is the combined use of anatomical and functional methods, since a single test technique has insufficient sensitivity and specificity (Ricke, 2000; Kaltsas, 2004).

The most commonly used diagnostic techniques for NETTs include anatomical examinations such as ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS) (Kaltsa, 2004).

The image of NETTs in CT is non-specific and takes the form of a large, clearly delimited tumor mass with a heterogeneous signal intensity. CT allows the identification of possible cystic lesions, necrosis, hemorrhage or hemorrhage within the tumor (Xiang, 2010). In an MRI scan, thymic tumors take the form of emerging tumor masses, which also show a heterogeneous signal intensity and allow the detection of cystic lesions. MRI scan is crucial in excluding possible tumor infestation into adjacent mediastinal structures (Berman, 2020; Xiang, 2010).

In turn, functional techniques, scintigraphic studies are used, which are based on a specific connection of synthetic somatostatin analogues labeled 111In or 99mTc with transmembrane-helial receptor protein – scintigraphy of somatostatin receptor (SRS) (Krenning, 1989). The somatostatin receptors' presence in the neoplastic tissue justified the use of 111-Indium-diethylenetriamine pentaacetic acid-D-phenyl-alanine-octreotide (Octreoscan) scintigraphy, both in preoperative and in follow-up settings (Filosso, 2017).

Good quality imaging studies are the fundamental elements in establishing the starting point of primary neuroendocrine tumors of the thymus in assessing their stage. This is essential in determining surgical management, tracking response to therapy and prognosis (Plöckinge, 2005).

TREATMENT OF THE PRIMARY NEUROENDOCRINE TUMORS OF THE THYMUS

Primary neuroendocrine tumors of the thymus are rare yet very aggressive tumors, which grow relatively slowly. In almost 80% of the cases, they are malignant. NETTs very often infiltrate adjacent tissues. Local recurrence may occur many years later. They are more frequently diagnosed in men in the fourth and fifth decade of life. Nearly half of the cases are associated with endocrinology, such as Cushing's syndrome or acromegaly (Pier Luigi Filosso, 2017).

Completeness of resection is believed to be the strongest prognostic factor in the prognosis of this disease (Filosso, 2014). It has been found that patients in early stage of NETT survived longer and developed recurrences less frequently (Filosso, 2015). Furthermore, tumor size and metastatic development are also important in prognosis. According to previous studies, tumors with associated endocrinopathies also act more aggressively than tumors without them (Rabinowitcz, 2006). It was observed that patients with NETT and Cushing's syndrome or MEN-1 syndrome had a higher mortality rate than those without paraneoplastic syndromes (Wick, 1980).

Patients with NETT should be routinely referred to experienced centers and multidisciplinary facilities. For NETT, surgery to reduce the tumor mass is recommended to alleviate clinical symptoms resulting from the secretory activity of the tumor. These tumors respond poorly to radiotherapy. The treatment of choice is surgery because almost 80% of thymic NETT cases behave malignantly (Moran, 2000). Complete resection of the tumor along with the involved mediastinal structures should always be sought. The preferred approach for NETT resection is through a median sternotomy. For advanced tumors, anterior thoracotomy, lateral thoracotomy, posterior-lateral thoracotomy, alone or in combination with sternotomy (combined access) can be used, which provide good exposure of the entire mediastinum and pleural space (Huang, 2008). Despite this, these tumors can often infiltrate adjacent structures and cause distant metastasis and recurrence, making their complete resection sometimes difficult and their prognosis poor (Pier Luigi Filosso, 2017).

NETT recurrences can be local, occurring in the anterior mediastinum, regional, present within the chest, or distant, occurring outside the chest or in the case of intrapleural nodules. An aggressive surgical approach if complete resection of the recurrence is possible and postoperative RT is thought to be effective in recurrent NETTS and to increase survival. (Sakuragi, 2002). For advanced NETT, induction chemotherapy (or CT + RT) has been used to reduce tumor size, increasing the likelihood of R0 resection (radical resection), although studies do not clearly define the effect of such a process. (Pier Luigi Filosso, 2017). Postoperative radiotherapy (or CT + RT) is also used for incomplete resections. Based on the reported cases, the medium-term survival in patients with NETT was quite good, especially in the case of complete surgical resection.

When surgical treatment is not possible, pharmacotherapy with somatostatin analogues, a hormone that inhibits secretory and cell proliferative processes, can be used. Somatostatin analogues are very well tolerated and usually relieve discomfort resulting from the

secretory function of tumors (Dasari, 2017; Halperin, 2017; Davar, 2017).

A form of molecularly targeted therapy, peptide receptor radionuclide therapy (PRRT), appears to be very effective in the systemic treatment of metastatic thymic neuroendocrine tumors. PRRT is performed using a somatostatin analogue similar to octreotide, absorbed by the tumor, coupled to a radionuclide usually 177 lutetium and 90Itr emitting beta radiation that kills the tumor cells (Pier Luigi Filosso, 2017).

In order to reduce the tumor mass of metastases, thermoablation techniques are used, i.e. destroying cells with high temperatures obtained by laser or radiofrequency. In some patients with NET tumors, characterized by a high capacity for rapid cell division, classical chemotherapy is also used (Dasari, 2017; Halperin, 2017; Davar, 2017).

In MEN1 patients in whom NETT is a major cause of death, several prophylactic thymic resections at the time of parathyroidectomy using the same surgical access are suggested to reduce the risk of NETT (Teh,1998) (Trump, 1996).

As there is a high risk of recurrence or development of distant metastases in patients with NETT, close and lifelong follow-up of the patient is required. It is suggested to perform a chest CT every 6 months for the first 3 years (Pier Luigi Filosso, 2017).

THE IMPORTANCE OF EMPERIPOLESIS IN THE CONTEXT OF DEVELOPING FUTURE DIAGNOSTIC AND TREATMENT METHODS

In terms of diagnostics, it seems appropriate to conduct extensive research on a large population of neoplastic cells of neuroendocrine origin in terms of the occurrence of the phenomenon of emperipolesis. Based on the various studies and descriptions of clinical cases cited earlier, we conclude that there is a likelihood of a significant correlation between the number of cells in emperipolesis and a specific type of cancer. Furthermore, it is noteworthy that the proportions between different types of cells can serve as an indicator of a given tumor development and progression. It may be important to observe cells in the state of emperipolesis in a microscopic image and find the relationship between the occurrence of a specific image of cells and frequent detection of a specific tumor.

The use of lymphocytes in targeted therapy is very promising (Goswami, 2019). T lymphocytes tend to bind to antigens of cancer cells, which may be crucial for introducing therapeutic substances into cancerous cells, not into healthy ones. Targeted therapy can then only cover diseased cells, leaving healthy cells intact.

In biotechnology, great opportunities are attributed to the importance of liposomes as potential carriers of anti-cancer drugs (Temidayo, 2018). If the process of emperipolesis were to be explored even more and we would get an answer to the question of what induces emperipolesis, then one can try to construct a liposome that would resemble a lymphocyte externally, induce emperipolesis and thus deliver the drug to the inside of cancerous cells. Such a solution could be used locally or systemically if there is a risk of neoplastic metastases, since the outer surface of the liposome would have specific receptors targeting specific tumor epitopes distributed throughout the body.

A slightly different method could be to modify T lymphocytes by introducing specific drugs inside them and then using it in molecularly targeted therapy. This would save time and the biotechnological construction of the receptors would not be necessary, as we would use the receptors already present on the T lymphocytes.

Moreover, radioisotope therapy can be used in the treatment of neuroendocrine tumors of the thymus (Iskanderani, 2018). It is a molecularly targeted therapy in which a specially selected peptide, having the property of attaching to a cancer cell, is combined with a small amount of radioactive material to form together a drug (radiopharmaceutical) called a radiopeptide (Kolasińska-Ćwikła, 2018). After the injection into the patient's bloodstream, radiopeptide travels with the blood, reaches the tumor and attaches to the cancer cells, providing them directly with a therapeutic dose of radioisotopic radiation. The tumor absorbs both the drug and the radionuclide, and the emitted beta radiation particles kill cancer cells. The most effective radionuclides currently used are 177Lutet and 90Yttr.

SHORT CONCLUSION

Emperipolesis is a rare biological phenomenon, in which a cell penetrates another living cell. Emperipolesis is often described in relation to the thymus gland, however the precise mechanisms underlying this process are still elusive. In this publication we have reviewed previous findings and determined the importance of emperipolesis in tumors formation and progression.

Lymphatic emperipolesis may occur in thymic epithelia-reticular cells. It is crucial to clarify the relationship between the presence of a particular cell image during emperipolesis and the detection of a particular type of cancer. Among available diagnosing techniques, imprint smear is an effective and quick method for detecting emperipolesis.

Thymic neuroendocrine tumors (NETTs) are rare tumors with high aggressiveness that present many non-specific symptoms. Diagnostic techniques that are most commonly used in neuroendocrine tumors assessment are ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS). The treatment of choice is surgery. The completeness of resection is the strongest prognostic factor, nevertheless PRRT appears to be very effective during therapy. Targeted therapy can cover only diseased cells, leaving healthy cells intact. The use of modified T lymphocytes in targeted therapy by introducing specific drugs inside them is an emerging and very promising method in treating cancer.

There is still a lot to uncover regarding emperipolesis, especially in terms of using this phenomenon in the therapy and treatment of cancer. An interesting approach would be to construct the liposome that delivers the drug to the inside of cancerous cells. The combination of the well-known treatment methods with not yet fully understood em-peripolesis, may open up new possibilities especially in the treatment of neuroendo-crine tumors of thymus.

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Insulinoma-associated protein 1 (INSM1) – new nuclear marker of neuroendocrine differentiation with high sensitivity and specificity in immunohistochemical diagnostics of neuroendocrine neoplasms – review

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ABSTRACT

Diagnostically difficult cases of neuroendocrine neoplasms require the use of markers of neuroendocrine differentiation. However, even the use of traditional neuroendocrine markers such as synaptophysin, chromogranin, and CD56 yields negative results in 10% to 25% of high-grade neuroendocrine tumors. Insulinoma-associated protein 1 (INSM1) is a novel nuclear marker of neuroendocrine differentiation. In terms of structure, INSM1 is a zinc-finger transcription factor. INSM1 (formerly IA-1) contains five zinc-finger motifs. INSM1 expresses transiently in embryonic neuroendocrine tissues. In adult tissues INSM1 has been identified in multiple tumors of neuroendocrine or neuroepithelial origin. INSM1 is a strong nuclear marker of neuroendocrine differentiation with high sensitivity and specificity. The results of the research analysed in this paper indicate that INSM1 can be very useful in the diagnostics of neuroendocrine neoplasms of the lung, gastrointestinal tract, pancreas, head and neck, uterine cervix, and Merkel cell carcinoma. In order to be included in the review, articles from PubMed (NCBI), Google Scholar, Web of Science and Scopus archive had to fit the following criteria:

- they had to be original articles, case studies and reviews connected with the following key words: neuroendocrine neoplasms, well-differentiated neuroendocrine tumors, poorly-differentiated neuroendocrine carcinomas, INSM1, traditional markers such as chromogranin, synaptophysin and CD56;
- they had to be written in English;
- they had to be published between 1992 and 2020, as the first article about insulinoma-associated protein 1 was written by Goto et al. in 1992.

INTRODUCTION

The term *neuroendocrine system* was introduced in the second half of the 20th century. The neuroendocrine system covers interactions between the nervous system and a variety of endocrine glands such as: the pituitary gland, the thyroid gland, the parathyroid gland, the adrenal gland, the ovaries and testes, the endocrine pancreas, the pineal gland, the gastrointestinal endocrine system, and the respiratory endocrine system. The endocrine/neuroendocrine cells found in these organs and systems synthesize and secrete a number of hormones that have key influence on the metabolism of the body through the interaction of these hormones with target tissues in response to stress and injury. These hormones are also involved in the control of a number of life processes such as growth, development, absorption of nutrients, energy, metabolism, water and electrolyte balance, reproduction, birth, and lactation. The endocrine/neuroendocrine cells appear in the early stages of development and are characterized by a unique pathway of differentiation. According to Lan et al., abnormal differentiation and/or deregulation of these endocrine/ neuroendocrine cells appearing in the pituitary gland, the thyroid gland, the parathyroid gland, the adrenal gland, the ovaries and testes, the endocrine pancreas, the pineal gland, the gastrointestinal endocrine system, and the respiratory endocrine system may lead to the development of neuroendocrine tumors that have a profound effect on the body′s metabolism (Lan, 2009). However, the term neuroendocrine neoplasms (NENs) includes not only tumors developing in the above mentioned organs and systems. Neuroendocrine neoplasms occur throughout the body, in all body organs, including paraganglia and soft tissue (Choi, 2018; Delaloge, 2000; Egashira, 2018; Fujino, 2015; Ramalingam, 2016; Weed, 2003). This prompted the participants of the 2017 WHO conference to accept the term neuroendocrine neoplasms for approval in relation to the classification of the types of tumors mentioned above. According to Rindi et al. the term "neuroendocrine neoplasms"

is the best at "encompassing all tumor classes with predominant neuroendocrine differentiation, including both well and poorly differentiated forms" (Rindi, 2018). Moreover, the authors stated that the "key features defining these neoplasms at any specific anatomic site are, above all, multiple anatomic sources (neural structures, endocrine organs and/or neuroendocrine cells), morphology, and the expression of markers of neuroendocrine differentiation (general and specific)". The expression of neuroendocrine markers may fundamentally differ in different anatomic sites, and at the same time, expression depends on the degree of differentiation. Therefore, different general neuroendocrine markers to define neuroendocrine differentiation are currently applicable in different organs and systems (e.g. only chromogranin and synaptophysin in the gastrointestinal system and pancreas, while chromogranins, synaptophysin, and CD56 in the lung) (Rindi, 2018). According to the latest WHO classification from 2017, neuroendocrine neoplasms include well-differentiated neuroendocrine tumors (NETs) designated carcinoid tumors in some systems, as well as poorly-differentiated neuroendocrine carcinomas (NECs), including two separate morphologic types defined as small cell neuroendocrine carcinoma and large cell neuroendocrine carcinoma (Kim, 2016, Lloyd, 2017, Rindi, 2018). The above classification was accepted and adopted by the American Joint Committee on Cancer (8^{th} edition) and the current College of American Pathologists guidelines (Amin, 2017; Burgart, 2020; Shi, 2017; Shi, 2020). Moreover, it was proposed that well-differentiated neuroendocrine tumors be classified in three tiers as G1, G2 or G3 which reflects low-grade, intermediate-grade, and high-grade (Rindi, 2018). Unlike NETs, NECs are always high grade (G3). On the other hand, three grading parameters such as: mitotic count and/or Ki-67 cell labeling index, and/or the presence or absence of necrosis are prognostic (Rindi, 2018). For this reason, the above division of neuroendocrine neoplasms is based on genetic evidence at specific anatomic sites and differences in epidemiology, histology, clinical course and prognosis. NETs, belonging to the family of well-differentiated neoplasms, have potential to metastasize or invade the adjacent tissues depending on tumor site, type, and grade (Klimstra, 2010; Klöppel, 2017). In turn, NECs are characterized by a high degree of malignancy, a very rapid and aggressive course and a poor prognosis. NENs are among a relatively rare group of tumors in the population, ranging from 2.5 to 5 cases per 100,000 people per year (Rodriguez, 2018; Rosenbaum, 2015). However, in recent years, a gradual increase in the incidence of neuroendocrine neoplasms has been observed both in the United States and in other countries (Dasari, 2015; Hallet, 2015; Hauso, 2008). The incidence of NENs has been increasing at all sites, stages and grades (Dasari, 2015), with the main sites of development in the human body affecting the gastrointestinal system and respiratory system (Oronsky, 2017). NENs occur in the population in all age groups, but the highest number of NENs is observed in patients aged 65 years and older (Dasari, 2015).

According to the latest WHO classification from 2017, neuroendocrine neoplasms have epithelial or neuronal/neuroectodermal origin, and share major morphological and protein expression signatures depending on differentiation, despite their different localization in the body (Rindi, 2018). While NENs are characterized by a diverse spectrum of proteins, many of these proteins are identical to proteins present in normal cells, organs or systems with different anatomical localization. Different localization in the body and origin make that NENs a heterogenous group of tumors, yet they share some common features, including presence of neurosecretory granules and typically showing a characteristic histology and immunoprofile (Rindi, 2018). The characteristic markers of general neuroendocrine differentiation occurring in NENs include chromogranin A, chromogranin B, and synaptophysin, as well as site specific markers such as hormones and transcription factors (Inzani, 2017). The following immunohistochemical markers of neuroendocrine differentiation are traditionally used in immunohistochemical diagnostics: synaptophysin, chromogranins and CD56. These immunohistochemical markers are characterized by a relatively low sensitivity and specificity. Research results indicate that synaptophysin shows expression in only 41% to 75% of small cell lung carcinoma (SCLC) and from 58% to 85% of large cell lung carcinoma (LCNEC) cases, chromogranin shows expression in only 23% to 58% of SCLC and 42% to 69% of LCNEC and CD56 showed expression from 72% to 99% of SCLC and 72% to 94% of LCNEC (Hamanaka, 2014; Jiang, 1998;

Kaufmann, 1997). Since none of the above immunohistochemical markers are sufficiently sensitive and specific, they must be used in immunological diagnostics as a group. This creates a situation where diagnostics is overly complicated and expensive. Therefore, the search for a single immunohistochemical marker with high specificity and sensitivity that could be used in the diagnostics of neuroendocrine neoplasms has been going on for many years. Insulinoma-associated protein 1 (INSM1) may fulfill these expectations.

NEW INSIGHT

STRUCTURE AND FUNCTION OF INSULINOMA-ASSOCIATED PROTEIN 1

Insulinoma-associated protein 1 is a zinc-finger transcription factor. At the same time, the protein structure of INSM1 is highly conserved among homologues of different species. INSM1 (formerly IA-1) contains five zinc-finger motifs. Based on the deduced protein sequence, INSM1 can be divided into two major domains. The aminoterminal domain (aa 1-250) contains a high percentage of proline, glycine, and alanine residues. Prolinerich (20-30%) sequences occur in many mammalian transcription factors and serve as protein-protein interacting domains that mediate both transcriptional activation and/or repression (De Caestecker, 2000; Zilfou, 2001). The dibasic amino acids are cleavage recognition sites for processing peptide hormone precursors such as insulin, glucagon, somatostatin and pancreatic polypeptide. An α-amide group is common to many bioactive neuroendocrine peptides. The carboxyl-terminal sequence (aa-251-510) contains five putative Cys2-His2-type zinc-finger motifs. These five zinc-finger motifs are symmetrically spaced at the carboxy terminus. Two tandem repeated zinc-finger motifs from either end are spaced by 45/46 aa from the middle zinc finger (Lan, 2009). The structural features of INSM1 indicates that INSM1 is a zinc-finger DNA-binding protein. INSM1 functions as a transcriptional repressor that simultaneously regulates entry into the cell cycle and controls expression of a neuroendocrine phenotype (Lan, 2009). Moreover, INSM1 is directly responsible for the transcription of synaptophysin and chromogranin (Fujino, 2015). In contrast, INSM1 is regulated by neurogenin 3 (Mellitzer, 2006).

INSM1 shows expression mainly in normal fetal neuroendocrine tissues and tumors of neuroendocrine origin regardless of age. In the fetal period, INSM1 is predominantly expressed in the nervous system in mammals, and plays an important role in early embryonic neurogenesis (Lan, 2009). Moreover, in the fetal period, INSM1 plays an important role in the development of normal neuroendocrine cells in various tissue throughout the body, mainly in the pancreas, digestive system and central nervous system (Gierl, 2006; Goto, 1992; Lan, 1993; Lan, 2009; Xie, 2002). It was found that INSM1 affects both terminal cellular differentiation and cellular proliferation in the pancreas (Gierl, 2006; Osipowich, 2014; Parent, 2008; Zhang, 2012; Zhu, 2002), enteroendocrine cells (Gierl, 2006), the autonomic nervous system (Widner, 2008), the central nervous system (Farkas, 2008; Jacob, 2009), olfactory epithelium (Rosenbaum, 2011), and the pituitary gland (Welcker, 2013). Moreover, INSM1 regulates downstream target genes and exhibits extranuclear activities associated with multiple signaling pathways, including Sonic Hedgehog, PI3K/AKT, MEK/ERK, ADK, p53, Wnt, histone acetylation, LSD1, cyclin D1, Asc1, and N-myc (Chen, 2018; Chen, 2019). However, a disadvantageous phenomenon is that INSM1 expression declines with age (Goto, 1992).

What is interesting, however, is that when it comes to tumors, the presence of INSM1 can be found in a number of tumors with neuroendocrine differentiation, such as pheochromocytoma (Sandgren, 2010), medullary thyroid carcinoma, pituitary adenoma (Goto, 1992), hypothalamic hamartoma (Parent, 2008), retinoblastoma, small cell lung carcinoma (Amelung, 2010; Goto, 1992; Lan, 1993; Taniwaki, 2006), and medulloblastoma (Breslin, 2002; De Smaele, 2008; Gilbertson, 2004; Pomeroy, 2002). INSM1 was found not only in human tumors but also in mice and rats (Farkas, 2008; Jacob, 2009; Kawaguchi, 2008; Xie, 2002). Initially, it was thought that INSM1 did not appear in the normal tissue of adults (Breslin, 2003; Duggan, 2008; Gierl, 2006; Goto, 1992; Welcker, 2013; Widner, 2008; Zhu, 2002). However, further research has shown INSM1 expression in normal adult cells such as neuroendocrine cells present in the gastrointestinal tract, pancreatic tract, bronchopulmonary system, adrenal medullary tissues, and in occasional individual cells in nonneoplastic prostate glands

(Ames, 2018; Rosenbaum, 2015; Yoshida, 2018).

INSM1 is encoded by the insulinoma associated-1 (IA-1) gene of cDNA. This gene was first identified by Goto et al. in 1992 in human pancreatic insulinoma tissues and murine insulinoma cell lines, which influenced its name (insulinoma associated protein 1) (Goto, 1992). However, the localization of the *INSM1* gene at the start arm of chromosome 20 was revealed by Lan et al. two years later (Lan, 1994). Research conducted on human lung cancer cell lines has shown that *INSM1* gene expression occurs in small cell lung carcinoma and carcinoid tumors, while expression of this gene does not occur in non-small cell lung carcinoma (Lan, 1993). Subsequent studies have shown that the expression of *INSM1* gene is not limited to small cell lung carcinoma but also occurs elsewhere of the body, including neuroendocrine tumors of the gastrointestinal tract, cervical cancer, prostate cancer, pheochromocytoma, medullary thyroid carcinoma, insulinoma, or pituitary tumors (De Smaele, 2008; Gilbertson, 2004; Goto, 1992; Parent, 2008; Pomeroy, 2002; Sandgren, 2010; Xin, 2018).

INSM1 AS IMMUNOHISTOCHEMICAL AND MOLECULAR MARKER

INSM1 shows high expression in tumors of neuroendocrine origin, with INSM1 expression significantly increased in neoplastic tissue compared to non-neoplastic tissue (Doxtader, 2018; Lan, 2009; Nakra, 2019; Rodriguez, 2018; Rosenbaum, 2015). Moreover, research conducted by many authors has confirmed that INSM1 is a strong nuclear, immunohistochemical marker of neuroendocrine differentiation in neoplastic human tissues (González, 2019; Rosenbaum, 2015; Roy, 2019; Staaf, 2020; Viswanathan, 2019). For this reason, INSM1, the only available nuclear neuroendocrine marker, is increasingly used in immunohistochemistry diagnostics (Ames, 2018; Kuji, 2017; Rooper, 2018; Rosenbaum, 2015; Xin, 2018). INSM1 in immunohistochemical staining gives a positive nuclear reaction, in contrast to synaptophysin and chromogranin, which show a granular cytoplasmic reaction. In turn, CD56 is both cytoplasmic or membrane positive.

REVIEW AND DISCUSSION

NEUROENDOCRINE NEOPLASMS IN THE LUNG

In one of the first large studies involving 111 primary thoracic neuroendocrine neoplasms (small cell carcinoma, large cell carcinoma, atypical carcinoid, typical carcinoid and mediastinal paraganglioma) and 156 non-neuroendocrine tumors (adenocarcinoma, and squamous cell carcinoma), the authors assessed immunohistochemistry sensitivity and specificity of INSM1 in surgical specimens and compared its performance to traditional neuroendocrine markers (synaptophysin, chromogranin and CD56) (Rooper, 2017). For this purpose, they used material from the surgical pathology archives from 1997-2017, but did not include thoracic mixed tumors in the study. In the presented study, the sensitivity of INSM1 for small cell lung carcinomas and large cell neuroendocrine carcinomas was significantly higher (94%, 91.3%) than the sensitivity of the panel of the three traditional neuroendocrine markers (74.4%, 78.3%). In addition, the authors found positive staining for INSM1 in all the atypical carcinoids, typical carcinoids and mediastinal paragangliomas. The sensitivity of INSM1 across all grades of thoracic neuroendocrine tumors was 96.4%, and significantly exceeded the sensitivity of the panel of traditional neuroendocrine markers (87.4%). However, in non-neuroendocrine tumors staining positive for INSM1, they observed only 3.3% of adenocarcinomas and 4.2% of squamous cell carcinomas.

In another large study, researchers examined a large series of whole-tissue sections of primary lung neoplasms (345), including 152 neuroendocrine tumors (64 small cell lung carcinomas, 24 large cell neuroendocrine carcinomas, 48 typical carcinoid tumors, 16 atypical carcinoid tumors), and 163 non-neuroendocrine tumors (130 adenocarcino-mas, 33 squamous cell carcinomas) for sensitivity and specificity of INSM1 (Mukhopadhyay, 2019). The analyzed material also included mixed tumors. In this study, the sensitivity of INSM1 for neuroendocrine neoplasms *as a group* (95%) was similar to synaptophysin and CD56 (98%, 97%), but higher than chromogranin (84%). In contrast, the specificity of INSM1 and chromogranin (97%, 98%) was higher than the specificity of synaptophysin and CD56 (90%, 87%). The sensitivity of INSM1 in small cell carcinoma was similar to the sensitivity of synaptophysin and CD56 (98%, 100% and

95%), but was higher than the sensitivity of chromogranin (83%). For large cell neuroendocrine carcinomas, similar sensitivity for CD56 and synaptophysin (92%, 88%) was observed, while the sensitivity of INSM1 and chromogranin was unquestionably less (75%, 46%). Except for one case of atypical carcinoid tumor, all carcinoid tumors were positive for INSM1, chromogranin, synaptophysin and CD56.

A third study looked at surgically resected 54 primary lung neuroendocrine tumors (including 24 small cell lung carcinomas, 23 large cell lung carcinomas, 5 typical carcinoid tumors and 2 atypical carcinoid tumors) as well as 623 non-small cell lung carcinomas (Staaf, 2020). There were also mixed tumors in the material studied. Here, the authors determined the diagnostic value of INSM1 in comparison to the previously used traditional neuroendocrine markers (CD56, synaptophysin and chromogranin A). They observed positive INSM1 staining in 39 cases of 54 pulmonary neuroendocrine tumors (72%) and in 6 cases of 623 non-small cell lung carcinomas (1%). On the other hand, a positive CD56 staining for primary lung neuroendocrine tumors and non-small cell lung carcinomas were 47 of 54 (87%) and 14 of 626 (2%), for synaptophysin 46 of 54 (85%) and 49 of 630 (8%), and for chromogranin A 30 of 54 (56%) and 6 of 629 (1%).

Other authors tested for whether INSM1 could be used in cytology (Cellient) cell blocks and whether these results correlated with surgical pathology specimens (Doxtader, 2018). The aim was to compare the sensitivity and specificity of INSM1 with the sensitivity and specificity of synaptophysin, chromogranin and CD56. The study was conducted on seventy-four primary lung neoplasms, including 52 primary lung neuroendocrine neoplasms (41 small cell lung carcinomas, 1 large cell neuroendocrine carcinoma, 10 carcinoid tumors) and 22 non-neuroendocrine primary lung tumors (11 adenocarcinomas, 9 squamous cell carcinomas, 1 mesothelioma, and 1 poorly differentiated non-small cell lung carcinoma). In 20 cases, INSM1 staining was performed simultaneously on paired surgical pathology specimens (biopsy or resection). The specimens tested positive for INSM1 in all 20 paired surgical pathology cases. However, in cytology cell blocks, positive INSM1 results were found in 48 of 52 cases of primary lung neuroendocrine neoplasms (92%), including 38 of 41 small cell lung carcinomas (93%), in one case large cell neuroendocrine carcinoma (100%) and in 9 cases out of 10 carcinoid tumors (90%). The specificity of INSM1 for primary pulmonary neuroendocrine neoplasms *as a group* was identical to the specificity of chromogranin (100%), but was higher than the specificity of synaptophysin (95%) and CD56 (95%).

In another study, the authors compared the diagnostic utility of INSM1, CD56, synaptophysin and chromogranin in the largest cohort (143) of pulmonary cytology cell blocks (11 typical carcinoid tumors, 11 atypical carcinoid tumors, 9 small cell lung carcinomas, 8 large cell neuroendocrine carcinomas, 9 squamous cell carcinomas and 95 adenocarcinomas) and the largest available material (563) of surgical specimens including 17 typical carcinoid tumors, 14 atypical carcinoid tumors, 8 small cell lung carcinomas, 10 large cell neuroendocrine carcinomas, 58 squamous cell carcinomas, 415 adenocarcinomas, 17 large cell carcinomas and 24 other tumor types (Viswanathan, 2019). These authors obtained sensitivity and specificity for INSM1 of 92.3% and 100% in the cytology cell blocks, while the sensitivity and specificity for INSM1 in the surgical specimens was lower (89.8%, 98.1%). The sensitivity and specificity for CD56 were 97.4% and 93.3% in the cytology cell blocks and 93.9% and 93.6% in the surgical specimens. The sensitivity and specificity for synaptophysin and chromogranin were significantly lower in both the cytology cell blocks and the surgical specimens.

In the next study, the authors performed manual immunohistochemistry on small biopsies of INSM1 and immunocytochemistry on direct smears of INSM1 on archival material from 60 patients diagnosed with small cell lung carcinoma in order to check the suitability of each of these methods in the diagnostics of this tumor (Nakra, 2019). Of these 60 patients, 37 were tested for INSM1 immunohistochemistry on small biopsies and 36 were tested for INSM1 immunocytochemistry on direct smears. The sensitivity for INSM1 immunohistochemistry (small biopsies) was 97% (36 of 37 cases), while the sensitivity for INSM1 immunocytochemistry (direct smears) was lower, only 91% (30 of 33 cases). Moreover, INSM1 reactions were performed on 10 cases of non-small cell lung carcinoma on spare direct smears and on small biopsies, obtaining 100% specificity (all cases were negative for INSM1).

In yet another study, the authors performed immunohistochemistry staining for INSM1 on cytology samples from 32 patients with neuroendocrine tumors of the lung (8) and tumors with neuroendocrine differentiation of lung origin (22 lymph node, 1 chest wall mass, 1 thyroid) (Rodriguez, 2018). All the neuroendocrine tumors used in the study were small cell carcinomas. The material taken was from multiple aspirations. INSM1 was positively identified in 31 of 32 cases (97%). In the control group of non-neuroendocrine tumors all 13 cases were negative for INSM1. The sensitivity of CD56 in small cell carcinoma was 96%.

NEUROENDOCRINE NEOPLASMS IN THE GASTROINTESTINAL TRACT AND PANCREATICOBILIARY TRACT

In a retrospective study covering the archive material from 2013-2015, the authors examined 30 patients with primary gastroenteropancreatic neuroendocrine neoplasms and their metastatic diseases in the liver in terms of INSM1 sensitivity assessment and compared it with the sensitivity of chromogranin-A and synaptophysin (Gonzalez, 2019). Moreover, they assessed the changes in the expression of these markers in the material from primary and metastatic diseases. Most of the cases studied were small intestine and neoplasms were present in ileum, duodenum, Meckel′s diverticulum, pancreas, stomach, rectum and caecum. All studied cases of primary gastroenteropan-creatic neuroendocrine neoplasms were reactive for INSM1 and synaptophysin (100%), while the sensitivity of chromogranin-A was weaker (97%). In the material from metastatic neoplasms, sensitivity of INSM1 was weaker (94%) than the sensitivity of synaptophysin (100%) and chromogranin-A (97%). The specificity of INSM1 (96%) was comparable to the specificity of chromogranin-A (97%), and higher than that of synaptophysin (54%).

In turn, other authors compared the sensitivity and specificity of INSM1 with other neuroendocrine markers (synaptophysin, chromogranin and CD56) and the performance of the antibody according to site and differentiation of the tumor (Rodriguez, 2018). This study was performed using 134 specimens, including 91 neuroendocrine tumors with neuroendocrine features (taken from pancreas, liver, gastric and perigastric mass, abdomen, parotid gland, and other organs such as: lymph node, lung, soft tissue, vertebra, buttock, soft tissue of vagina, and pelvic wall). In this material, INSM1 showed a sensitivity of 99% and a specificity of 97%, while CD56 had a sensitivity only slightly lower (95.5%), but the specificity was very low (69.2%). In contrast, chromogranin had the weakest sensitivity (82.5%), while synaptophysin had the weakest specificity (66.7%). In contrast, among 10 cases diagnosed as non-neoplastic lesions, only two cases (pancreatic neuroendocrine islet cells and benign adrenal cells) were positive for INSM1.

In another study, the authors assessed the sensitivity and specificity of INSM1 in material covering 110 cases of primary neuroendocrine neoplasms of the gastrointestinal tract, appendix, and pancreas (McHugh, 2020). At the same time, they performed a sensitivity and specificity check of synaptophysin, chromogranin, CD56 and Ki67. INSM1 was positive in 89 of 110 (80.9%) primary gastrointestinal, appendiceal and pancreatic neuroendocrine neoplasms, while synaptophysin was positive 99.1%, chromogranin 88%, CD56 95.3%. In contrast, the specificity of INSM1 (95.7%) was higher than that of synaptophysin (86.0%), chromogranin (87.3%), and CD56 (86.0%).

Other authors studied INSM1 in conjunction with chromogranin, synaptophysin, and CD56 in 36 appendiceal adenocarcinoma ex-goblet carcinoid (21 primaries, 15 metastases) (Yang, 2019). In primary adenocarcinoma ex-goblet carcinoid, they obtained positive results for INSM1 62%, for chromogranin 86%, for synaptophysin 86% and for CD56 47%. In contrast, metastatic adenocarcinoma exgoblet carcinoid showed staining for INSM1 53%, for chromogranin 73%, for synaptophysin 80% and for CD56 21%.

NEUROENDOCRINE NEOPLASMS OF PANCREAS

In a retrospective study, the authors examined the usefulness of INSM1 for identifying pancreatic neuroendocrine tumors in 26 cell blocks and 29 surgical resections (Kim, 2020). Additionally, they performed INSM1 staining in other primary pancreatic tumors such as solid pseudopapillary neoplasms (14 cases), 11 acinar cell carcinomas and 21 pancreatic ductal adenocarcinomas. They obtained in all 55 cases of pancreatic neuroendocrine tumors a positive

nuclear test for INSM1, both in cell blocks and surgical resections (100% sensitivity), while sensitivity of synaptophysin was 97%, chromogranin 92%, and CD56 85%.

In turn, other authors assessed the expression of INSM1 in 14 cytology specimens obtained from endoscopic ultrasound-guided fine needle aspiration cytology during diagnostics of pancreatic neuroendocrine tumors (Takase, 2018). These authors used cytological specimens from 15 cases diagnosed as pancreatic ductal adenocarcinoma as a control group. In all 14 pancreatic neuroendocrine tumor cases, INSM1 showed expression in the tumor cells (100% sensitivity). In the control group, these authors observed INSM1-expressing cells within the adenocarcinoma cell cluster, but found no expression of INSM1 in the pancreatic duct cells or acinar cells.

The presented study was aimed at detected INSM1, chromogranin, synaptophysin and neural cell adhesion molecule immunohistochemically, in 25 cases of pure pancreatic neuroendocrine tumors and 2 mixed adenoneuroendocrine carcinomas (Tanigawa, 2018). As a control group, they used 5 cases of solid-pseudopapillary neoplasm, 7 cases of acinar cell carcinoma, and 15 cases of pancreatic ductal adenocarcinoma. These authors found the nuclear expression of INSM1 in all pure pancreatic neuroendocrine tumors (100% sensitivity). In 2 cases of mixed tumor the neuroendocrine carcinoma component was positive for INSM1, while the adenocarcinoma component was negative for INSM1. All control cases were negative for INSM1, while they were positive for synaptophysin.

NEUROENDOCRINE NEOPLASMS OF SKIN

In one study, the authors assessed INSM1 staining on 56 cases of Merkel cell carcinoma (47 primary tumors and 9 nodal metastases) (Lilo, 2018). All 56 cases of Merkel cell carcinoma showed expression of INSM1 (100% sensitivity). In contrast, synaptophysin, cytokeratin and chromogranin in the same material had expressions of 96%, 92% and 32%. In the control group (50 cases included various non-Merkel cell carcinoma neoplasms), no positive staining for INSM1 was found in any case.

In turn, other researchers developed their own dual immunohistochemistry protocol for INSM1/ cytokeratin 20 to detect dual expression of keratin and INSM1 on 15 small samples taken from Merkel cell carcinoma (Rush, 2018). They detected INSM1 in 14 of 15 specimens carrying a diagnosis of Merkel cell carcinoma (93% sensitivity). On the other hand, one specimen that was negative for INSM1 was also negative for cytokeratin and chromogranin, with only focal positivity for synaptophysin. Moreover, they checked the sensitivity of INSM1 in three other specimens of cutaneous neuroendocrine carcinoma (non-Merkel cell carcinoma) and obtained 100% sensitivity for INSM1. However, of the 8 cutaneous non-neuroendocrine neoplasms tested, only one tested positive for INSM1.

NEUROENDOCRINE NEOPLASMS OF THE HEAD AND NECK

Researchers performed INSM1 immunohistochemistry on 97 neuroendocrine tumors and 626 non-neuroendocrine tumors across all histologic grades and anatomic subsites of the head and neck (Rooper, 2018). These authors obtained the sensitivity of INSM1 99.0%, with a positive result for INSM1 they observed in all types of head and neck neuroendocrine tumors (middle ear adenoma, pituitary adenoma, paraganglioma, medullary thyroid carcinoma, olfactory neuroblastoma, small cell carcinoma, large cell neuroendocrine carcinoma, and sinonasal teratocarcinosarcoma). These authors obtained 97.6% specificity for INSM1 in almost all non-neuroendocrine tumors.

NEUROENDOCRINE NEOPLASMS OF THE UTERINE CERVIX

In one study, the authors made an immunohistochemical assessment of conventional neuroendocrine markers (chromogranin, synaptophysin and neural cell adhesion molecule) and INSM1 by analyzing 37 cases of high-grade neuroendocrine carcinoma of the uterine cervix (Kuji, 2017). These authors obtained the highest sensitivity (95%) for INSM1, while sensitivity for both chromogranin and synaptophysin was 86% and for neural cell adhesion molecules only 68%.

In turn, other authors, examining malignant tumors with neuroendocrine differentiation from the gynecologic organs, assessed the expression

of INSM1, synaptophysin, chromogranin, CD56, orthopedia homeobox and achaetescute homolog 1 in 2 cases in the uterine cervix (Roy, 2019). They obtained 100% sensitivity for INSM1, 100% for synaptophysin, 100% for CD56, 50% each for chromogranin and achaetescute homolog 1, and negative for orthopedia homeobox.

NEUROENDOCRINE TUMORS OF THE PROSTATE

In this study, the authors checked the expediency of the use of INSM1 in the diagnostics of neuroendocrine tumors of the prostate (Xin, 2018). They performed immunohistochemical tests on 13 needle biopsies of primary small cell carcinoma of the prostate, 5 samples of mixed small cell neuroendocrine carcinoma-acinar adenocarcinoma obtained from prostatectomy and 2 cases of metastatic small cell carcinoma. These authors obtained positive results for INSM1 in 12 cases of primary small cell carcinoma (92.3%), while the reactions for synaptophysin (84.6%) and chromogranin (53.8%) were weaker. In the remaining 5 cases of mixed tumors and 2 metastatic tumors sensitivity of INSM1 was 100%, similarly for synaptophysin, while the sensitivity of chromogranin (80%) was weaker. The test of the specificity of INSM1 was performed on the material including benign prostatic hyperplasia and prostate adenocarcinoma, in most cases they did not find nuclear reactivity for INSM1.

In turn, Roy et al. assessed the usefulness of INSM1 in immunohistochemical diagnostics – 32 cases included malignant tumors with neuroendocrine differentiation from the gynecologic organs, including prostate gland (n = 6) (Roy, 2019). Out of 4 examined cases of prostate adenocarcinoma with neuroendocrine differentiation, they obtained a positive result for INSM1 in 25%. However, for synaptophysin and Cd56 they obtained a positive result in 50%, and chromogranin was negative in all cases.

OTHER RARE LOCALIZATION OF NEUROENDOCRINE TUMORS

NEUROENDOCRINE NEOPLASMS OF THE URINARY BLADDER

In the presented study, the authors assessed the immunohistochemical expression of INSM1 on 32 whole sections of small cell neuroendocrine carcinoma of the urinary bladder and compared INSM1 expression with synaptophysin, chromogranin and CD56 (Kim Jr, 2020). In 28 cases these authors obtained a positive result for INSM1, in 24 cases for CD56, in 19 cases for synaptophysin, and in 14 cases for chromogranins.

NEUROENDOCRINE NEOPLASMS OF THE BREAST

In another study, the authors compared the expression of INSM1, orthopedia homeobox, chromogranin, synaptophysin, CD56 and achaetescute homolog 1 in invasive mammary carcinoma (Roy, 2019). In the material studied, they found the strongest expression for achaetescute homolog 1 and synaptophysin (85.7%) and weaker for INSM1, chromogranin, and CD56 (71.4%). In contrast, the expression of orthopedia homeobox was negative.

PERIPHERAL NEUROBLASTIC TUMORS

In another study, the authors assessed the immunohistochemical profile of INSM1 in cases of peripheral neuroblastic tumors and compared INSM1 expression in these tumors to that seen in other embryonal neoplasms (non-neuroblastic tumors) (Wang, 2019). Nuclear expression of INSM1 was 78% in peripheral neuroblastic tumors, including in neuroblastomas 84%, in ganglioneuroblastomas 100%, and in ganglioneuromas 33%. In the non-neuroblastic tumors control group, these authors found INSM1 expression in rhabdomyosarcomas (50%), in nephroblastomas (32%), and in Ewing sarcomas (20%).

PRIMARY CENTRAL NERVOUS SYSTEM NEOPLASMS

Other authors checked INSM1 expression in primary central nervous system neoplasms (Ames, 2018). They obtained nuclear immunostaining for INSM1 in medulloblastomas (87%), while diffuse nuclear INSM1 immunostaining was observed in all central neurocytomas and pituitary adenomas. However, they found rare staining with INSM1 in other high-grade embryonal tumors and high-grade gliomas. These authors observed nuclear INSM1 staining in normal brain tissue only in early neuronal development, while they did not find nuclear INSM1 staining in adult normal brains, including areas of gliosis.

In typical cases, when the diagnostics of neuroendocrine neoplasms is not difficult, it is based on standard histologic and cytologic stains and there is no need to perform immunohistochemical testing (Mukhopadhyay, 2019). In diagnostically difficult cases, when the clinical picture of the disease and the histologic features of the examined tumor are not typical and differ from the accepted norm, immunohistochemical reactions are performed, thanks to which it is possible to identify the neuroendocrine differentiation, enabling the classification of neuroendocrine tumors. Currently, three conventional markers of neuroendocrine differentiation (synaptophysin, chromogranin, and CD56) are used in the histopathological diagnostics of neuroendocrine neoplasms, but the test result does not always give an explicit answer to the type of tumor present. This is due to the fact that synaptophysin is sensitive, but not specific enough, chromogranin is highly specific, while its sensitivity is very weak, and CD56 is highly sensitive, but due to its limited specificity it may stain a variety of non-neuroendocrine tumors. Even the use of a combination of these markers on surgical specimens or cytology specimens gives negative results in 10% to 25% of high-grade neuroendocrine tumors (Hamanaka, 2014, Maleki, 2012, Nicholson, 2002, Travis, 2015, Zheng, 2013). There is therefore a need to find a new neuroendocrine marker that would demonstrate both high sensitivity and specificity. The use of INSM1 in histopathological diagnostics of neuroendocrine neoplasms, which is the only nuclear neuroendocrine marker with high sensitivity and specificity so far, gives hope for a more accurate diagnosis in diagnostically difficult cases. However, the results of studies on the usefulness of INSM1 in the diagnostics of neuroendocrine neoplasms are not conclusive.

Neuroendocrine neoplasms are mainly located in the respiratory system and digestive system, with 25% of primary lung neoplasms being neuroendocrine tumors, 75% of which are mixed neuroendocrine tumors containing also a non-neuroendocrine component (Gustafsson, 2008). These tumors are characterized by very high mortality (Friedberg, 1997, Travis, 1998). Investigating all primary lung neuroendocrine neoplasms on surgical specimens Rooper et al. and Mukhopadhyay et al. obtained high sensitivity of INSM1 (96.4% and 95%) (Mukhopadhyay, 2019, Rooper, 2017). Similar results were also obtained by Doxtader et al. and Viswanathan et al. comparing the sensitivity of INSM1 of primary lung neuroendocrine neoplasms in cytology cell blocks (92%), (92.3%) with surgical specimens (100%), (89.8%) (Doxtader, 2018, Viswanathan, 2019). In contrast, the study completed by Staaf et al. on surgical specimens found much weaker sensitivity of INSM1 (72%), which may be due to the fact that they had a much smaller number of cases than the other authors (Doxtader, 2018, Mukhopadhyay, 2019, Rooper, 2017, Staaf, 2020, Viswanathan, 2019). In turn, Rooper et al. found a significantly higher sensitivity of INSM1 for neuroendocrine lung neoplasms *as a group* compared to each individual neuroendocrine marker (synaptophysin, chromogranin and CD56) (Rooper, 2017). None of the other authors observed statistically significant differences when comparing the sensitivity of INSM1 with the sensitivity of individual neuroendocrine markers (Doxtader, 2018; Mukhopadhyay, 2019; Staaf, 2020; Viswanathan, 2019). In their research, both on cytology specimens and surgical specimens, sensitivity of INSM1 for neuroendocrine lung neoplasms *as a group* was similar to synaptophysin and CD56, and statistically higher than chromogranin (Doxtader, 2018, Mukhopadhyay, 2019, Staaf, 2020. Viswanathan, 2019). Rooper et al. also found a significantly higher sensitivity of INSM1 compared to all three markers (synaptophysin, chromogranin and CD56) treated *as a group* (Rooper, 2017), while in the study by Mukhopadhyay et al. and Kriegsmann et al. sensitivity of INSM1 (95%, 76%) was weaker than the sensitivity of the traditional three neuroendocrine markers treated *as a group* (100%, 97%) (Kriegsmann, 2020, Mukhopadhyay, 2019). The observed differences may be due to the fact that the study by Rooper et al. excluded mixed neuroendocrine tumors from their material, while the other authors also examined mixed primary lung neoplasms with non-neuroendocrine component (Doxtader, 2018, Kriegsmann, 2020, Mukhopadhyay, 2019, Rooper, 2017, Staaf, 2020. Viswanathan, 2019).

Results obtained by Mukhopadhyay et al. regarding sensitivity of INSM1 for small cell lung carcinoma (98%) are comparable to the data reported by Rooper et al. (94.9%), Rosenbaum et al. (100%) and Fujino et al. in surgical specimens (100%), Doxtader et al. on cytology cell blocks (93%), Nakra et al. and

Rodriguez et al. on small biopsies (97%) and on cytology specimens (91%) (Fujino, 2015; Mukhopadhyay, 2019; Nakra, 2019; Rodriguez, 2018; Rooper, 2017; Rosenbaum, 2015). In material derived from carcinoid tumors Mukhopadhyay et al. observed a sensitivity of INSM1 of 98% (Mukhopadhyay, 2019). This result is similar to the result obtained by Fujino et al., Rooper et.al. and Rosenbaum et al. (100%) (Fujino, 2015; Rooper, 2017, Rosenbaum, 2015). In contrast, the sensitivity of INSM1 in relation to large cell neuroendocrine carcinoma described by Mukhopadhyay et al. (75%) was significantly lower than the results obtained by Rooper et al. (91.3%) (Mukhopadhyay, 2019; Rooper, 2017). It is difficult to explain the reason for such a large disparity between the two studies, as both authors used the same clone (A8) from the same company (Santa Cruz). Perhaps the reason may be the slight difference in methodology. The dilution of INSM1 (1:250) used by Mukhopadhyay et al. was weaker than in the Rooper study (1:200). In Mukho-padhyay's study, the antibody was dispensed manually. Mukhopadhyay et al. used Ventana's Optiview detection kit with the optional amplifer, while Rooper et al. used Ventana's UltraView detection kit.

Doxtader et al. observed that the specificity of INSM1 for pulmonary neuroendocrine neoplasms in cytology cell blocks was similar to the specificity of chromogranin (100%) and higher than the specificity of synaptophysin (95%) and CD56 (95%) (Doxtader, 2018). Similarly, Viswanathan et al. in cytology specimens found the same specificity for INSM1, synaptophysin and chromogranin (100%) and weaker specificity for CD56 (Viswanathan, 2019). In surgical specimens, Rooper et al. and Mukhopadhyay et al. observed high values of the specificity of INSM1 (96.2%), (97%), synaptophysin (96.8%), (90%) chromogranin (99.4%), (98%), and CD56 (93.7%), (87%) (Mukhopadhyay, 2019; Rooper, 2017). On the other hand, the specificity of INSM1 (87%) for primary neuroendocrine neoplasms was significantly higher compared to the three traditional neuroendocrine markers *as a group* (61%) (Mukhopadhyay, 2019). In addition, Rooper et al. found an upward trend in the specificity of INSM1 compared to the traditional panel of neuroendocrine markers, but it was not a statistically significant difference (Rooper, 2017).

The second most common site of neuroendocrine neoplasms is the digestive system, with primary neuroendocrine neoplasms having a different digestive tract localization that strongly influences the expression of INSM1. Gonzalez et al. found 100% sensitivity of INSM1 in primary gastroenteropancreatic neuroendocrine neoplasms and 94% sensitivity of INSM1 in metastatic gastroenteropancreatic neuroendocrine neoplasms (Gonzalez, 2019). Similarly, Rodriguez et al. observed 99% sensitivity of INSM1 in neuroendocrine tumors with neuroendocrine features in the digestive tract (Rodriguez, 2018). On the other hand, Rosenbaum et al. found significantly higher expression of INSM1 of midgut gastrointestinal neuroendocrine neoplasms with known metastases compared to those that had not yet metastasized (Rosenbaum, 2015). In turn, McHugh et al. who included the material from the appendix in the primary gastroenteropancreatic neuroendocrine neoplasms, obtained much weaker sensitivity of INSM1 (80.9%) compared to the results of Gonzalez et al. and Rodriguez et al. (Gonzalez, 2019; McHugh, 2020; Rodriguez, 2018). Also, Yang et al. who tested only primary appendiceal adenocarcinoma of ex-goblet cells obtained very poor sensitivity of INSM1 (62%) showing no differences compared to chromogranin, synaptophysin, and CD56 (Yang, 2019).

The results of tests by three independent teams on pure pancreatic neuroendocrine neoplasms showed 100% sensitivity of INSM1 (Kim, 2020, Takase, 2018, Tanigawa, 2018). Such high sensitivity concerned both cell blocks and surgical specimens, and it was higher than the 3 traditional neuroendocrine markers (synaptophysin 97%, chromogranin 92%, and CD56 85%). However, the disadvantage of INSM1 was the fact that in the case of pancreatic non-neuroendocrine tumors Kim et al. obtained positive staining in pancreatic solid pseudopapillary neoplasms, while Tanigawa et al. in the same tumor type observed no positive staining for INSM1 (Kim, 2020, Tanigawa, 2018). On the other hand, Takase et al. demonstrated the presence of INSM1 in pancreatic non-neuroendocrine tumors (pancreatic ductal adenocarcinoma within adenocarcinoma cell clusters) (Takase, 2018).

A rarer localization of neuroendocrine neoplasms is skin. When studying Merkel cell carcinoma, Lilo et al. observed 100% sensitivity and

specificity for INSM1 (Lilo, 2018). Similarly, high sensitivity for INSM1 (93%) in Merkel cell carcinoma was obtained by Rush et al. (Rush, 2018). Also, neuroendocrine neoplasms of the head and neck showed high sensitivity and specificity for INSM1 (99.0%) (97.6%) (Rooper, 2018). Similarly, in the uterine cervix Kuji et al. observed sensitivity for INSM1 (95%), and Roy et al. obtained 100% sensitivity for INSM1 (Kuji, 2017, Roy, 2019).

On the other hand, the results of research on the usefulness of INSM1 in the diagnostics of neuroendocrine neoplasm of the prostate, the urinary bladder, the breast, peripheral neuroblastic tumors or primary central nervous system neoplasms require further study, as they were based on single scientific reports.

CONCLUSION

The results of the analysed studies indicate that INSM1 is a strong nuclear marker of neuroendocrine differentiation with high sensitivity and specificity. In addition, the great advantage of nuclear staining with INSM1 is that it can be performed even on very small material samples containing a few cells, and at the same time it is easy to interpret the results both in surgical specimens and cytology specimens. INSM1 can be very useful in the diagnostics of neuroendocrine lung neoplasms as the firstline marker of neuroendocrine differentiation or in combination with synaptophysin or CD56. INSM1 also appears to be very useful in the diagnostics of pure pancreatic neuroendocrine neoplasms, neuroendocrine neoplasms of the digestive system (excluding tumors from the appendix), Merkel cell carcinomas, neuroendocrine neoplasms of the head and neck and the uterine cervix. The remaining locations of neuroendocrine neoplasms, due to the very small number of cases studied, require further research. Finally, INSM1 cannot be used to differentiate neuroendocrine neoplasms, because it stains both tumor cells in small cell lung carcinoma, large cell neuroendocrine carcinoma, typical carcinoid, atypical carcinoid and mediastinal paraganglioma.

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Analysis of brain tumor emission spectra in patients treated surgically

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ABSTRACT

Spectrophotometry is an instrumental technique that uses energy transitions in molecules for analytical purposes, caused by the absorption of electromagnetic radiation in the ultraviolet, visible or near-infrared range. Cancer cells, during their life or as a result of decay, produce their characteristic metabolites, which are able to absorb electromagnetic radiation in the ultraviolet or infrared range in various ways. The subject of this study was the spectrophotometric analysis of brain tumors that were removed during surgery and the determination of the correlation between the obtained results and the histopathological diagnosis obtained after the surgery. The study involved 50 adult patients, both sexes, treated surgically at the Department of Neurosurgery, Neurotraumatology and Pediatric Neurosurgery, Collegium Medicum im. Ludwik Rydygier in Bydgoszcz, Nicolaus Copernicus University in Toruń, due to a brain tumor. The research confirmed the truth of the assumption that the spectrophotometric evaluation is of clinical importance, which is also consistent with other results of research on spectrophotometry. Spectroscopic examination of the lesions (carried out in parallel with the histopathological examination) may also contribute to a more accurate diagnosis, and further treatment of lesions of a less advanced stage. The results obtained from these studies are expected to be a preliminary step towards the precise determination of the biology of brain tumors and an attempt to use fluorescent techniques in the early diagnosis of neoplastic lesions of the central nervous system.

INTRODUCTION

Brain tumors constitute about 2% of all cancers occurring in the world's population. Every year in Poland, a brain tumor is found in about 4,000 people. Clinical symptoms in patients can be divided into non-specific (associated with the increase in intracranial pressure) and specific (depending on the location of the tumor). Complementing the diagnosis in patients who are suspected of having a brain tumor are imaging examinations such as a computer tomograph (CT) and magnetic resonance imaging (MR). So far, scientific research on brain tumors did not give a full answer to the changes taking place under the influence of the carcinogenesis process. Histopathological examination of the tumor removed during surgery determines the biology of change.

We are currently looking for new methods that are able to dispel any diagnostic doubts. One of these methods may be spectrophotometric analysis of brain tumors. Fluorescence spectroscopy (fluorimetry, spectrofluorimetry) is a kind of electromagnetic radiation spectroscopy where the sample is analyzed using the fluorescence phenomenon induced by light in the visible or ultraviolet radiation range (Dowling, 2001; Saraswathy, 2009). Fluorescent methods of medical diagnosis are based on optical differences in the properties of healthy tissues and tissues changed in the process of cancer. Currently, spectroscopy is used, among others in biochemistry (in studies of the dynamics of enzymatic processes, the mechanism of vision and the course of photosynthesis), in crystallography, forensics (non-invasive examination of evidence, can be helpful in determining the authenticity of artistic works), in medicine (non-invasive *ex vivo* and *en vivo* studies on living tissues, for measuring blood glucose, diagnosing tissues, cellular tests, diagnosing cancerous changes and identifying the distribution of pigments in the skin). Over recent years, there has been significant progress in the biomedical sciences. Thanks to scientists, clinicians are provided with newer therapeutic methods and better and better diagnostic tools. Until now, doctors could obtain information about the patient's health status from an interview and a clinical trial – today they have many modern tools at their disposal. Modern diagnostics includes research at various biological levels: cells, tissues, organs and the entire organism. The most complicated apparatus allows you to look at numerous processes at the molecular level. Fluorescence spectroscopy is an example of such techniques. There are many reports in the literature on the use of photoluminescence for testing various tissues.

The apparatus for fluorescence measurements is called fluorimeters or spectrofluorimeters.

Cancer cells, during their lifetime or as a result of breakdown, produce their own characteristic metabolites, which in various ways are able to absorb electromagnetic radiation in the ultraviolet or infrared range. This method it can allow to assess the completeness of the surgical procedure and create conditions for the diagnosis of possible recurrence. The subject of this study is the spectrophotometric analysis of brain tumors removed during surgery and the determination of the correlation between the results obtained and the histopathological diagnosis obtained after the surgery. The results will be able to be compared with other studies on a given topic and will contribute to the broadening of knowledge about spectrophotometry, which in the future may allow better planning and more effective treatment and monitoring of the activity of these cancers. It is expected that the results obtained from these studies will be a preliminary step towards the exact determination of brain tumor biology and an attempt to apply fluorescent techniques in the early diagnosis of neoplastic lesions of the CNS.

ASSUMPTIONS AND AIM OF RESEARCH

Despite the high interest in spectrophotometry, there are still not many reports on the use of spectrophotometric methods in the case of brain tumors in the available world literature (Lin, 2001; Kast, 2014; Milad, 2013).

Main thesis: Spectrophotometric analysis of central nervous system tumors is of great clinical significance in determining prognosis, planning treatment and monitoring the activity of these tumors.

In order to verify the main assumption made, specific hypotheses were also put forward: In patients with central nervous system cancers, there is a relationship between emission spectrum and histopathological diagnosis.

MATERIAL AND METHODS

The study involved 50 adult patients, both sexes (27 females, 23 males), treated surgically in the Department of Neurosurgery, Neurotraumatology and Children's Neurosurgery, University Hospital No. 1 in Bydgoszcz due to solid brain cancer, from January 2013 to March 2015. A total of 57 fragments of brain tumors were recovered. The patient's qualifying diagnosis for the study was based on the interview, subject examination and neuroimaging. After surgery, histopathological diagnosis of brain tumor was established. Patients with impaired consciousness, making it impossible to express informed consent to participate in the research, were excluded from the study.

All persons qualified for the study (after having provided information about him) gave their written consent to participate in it. The research included spectrophotometric analysis of a brain tumor fragment taken during surgery. The material to be tested after sampling was placed in special containers with 0.9% NaCl, and then subjected to deep-freezing (temp about -30 degrees Celsius) (Richter, 2011; Moritz, 2012). Next, the research material was defrosted in accordance with GLP (Good Laboratory Practice), transferred to cuvettes and subjected to spectrophotometric analysis. In spectroscopic studies, measurements of fluorescence emission spectra were made. Excitation and fluorescence emission spectra were measured on an F-7000 spectrofluorimeter (Hitachi, Japan). Measurements of stationary autofluorescence spectra of brain tumor sections were made using a spectrofluorimeter for wavelengths of excitation 210 nm to 390 nm (measured every 20 nm – emission spectrum) and for light observations of 330 nm to 390 nm (measured at 20 nm – spectra excitation). The results in the form of diagrams were developed in the Origin 8. The statistical analysis of the collected material was carried out using the Statistica 12.5 package. Descriptive statistics and distribution characteristics were used to describe the variables. The intraoperatively collected material from the brain tumor was subjected to histopathological examination (histological type and tumor grade) in the Department of Clinical Pathomor-phology of Collegium Medicum. Ludwika Rydygiera in Bydgoszcz of the Nicolaus Copernicus University in Toruń.

RESULTS – ANALYSIS OF THE EMISSION

In the examined group of respondents, the most prevalent were those with CNS metastasis and with grade IV malignancy according to WHO.

The division in terms of the degree of malignancy was also made in a general way: malignant tumor (WHO III or IV), which was diagnosed in 25 tumor fragments, non-mali-

gnant (WHO I or II) in 13 tumor fragments, in 17 patients were found metastasis to the CNS.

The type of cancer was estimated in four general groups of intracranial tumors: gliomas, meningiomas, metastatic tumors and others.

The results of measurements of stationary spectroscopy were systematized depending on the histopathological diagnosis. The study material was divided into 3 groups: benign and intermediate grade malignant tumors, primary malignant tumors and metastatic tumors. Emission spectra were tested at light excitation in the wavelength range 250 nm, 270 nm, 290 nm, 310 nm, 330 nm, 350 nm, 370 nm, 390 nm.

EVALUATION OF BENINGN (WHO I) AND INTERMEDIATE (WHO II AND III) EMISSION LEVELS OF CNS TUMORS, MALIGNANT CNS TUMORS AND CNS METASTASES

EMISSION SPECTRUM WHEN EXCITED WITH 250 NM LIGHT OF CNS TUMORS

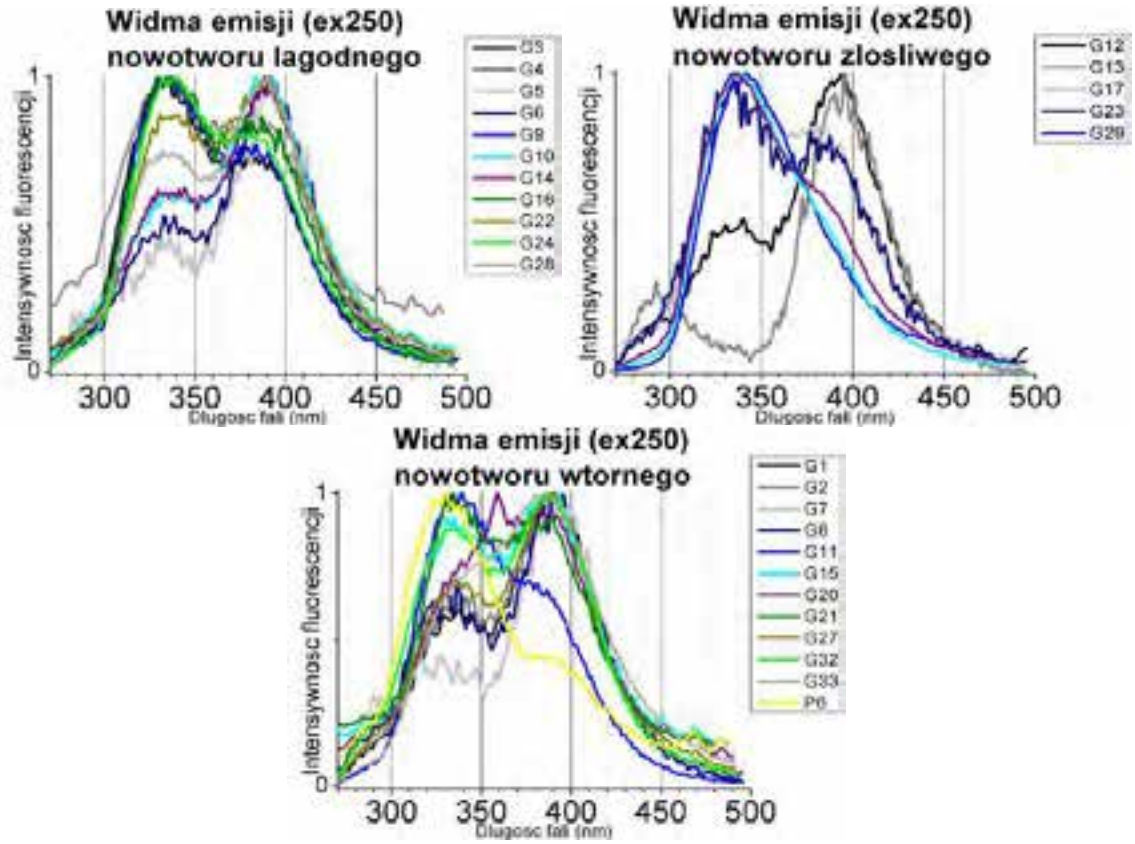

Figure 1. Normalized emission spectra at 250 nm excitation of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastases

EMISSION SPECTRUM WHEN EXCITED WITH THE 270 NM WORLD OF CNS TUMORS

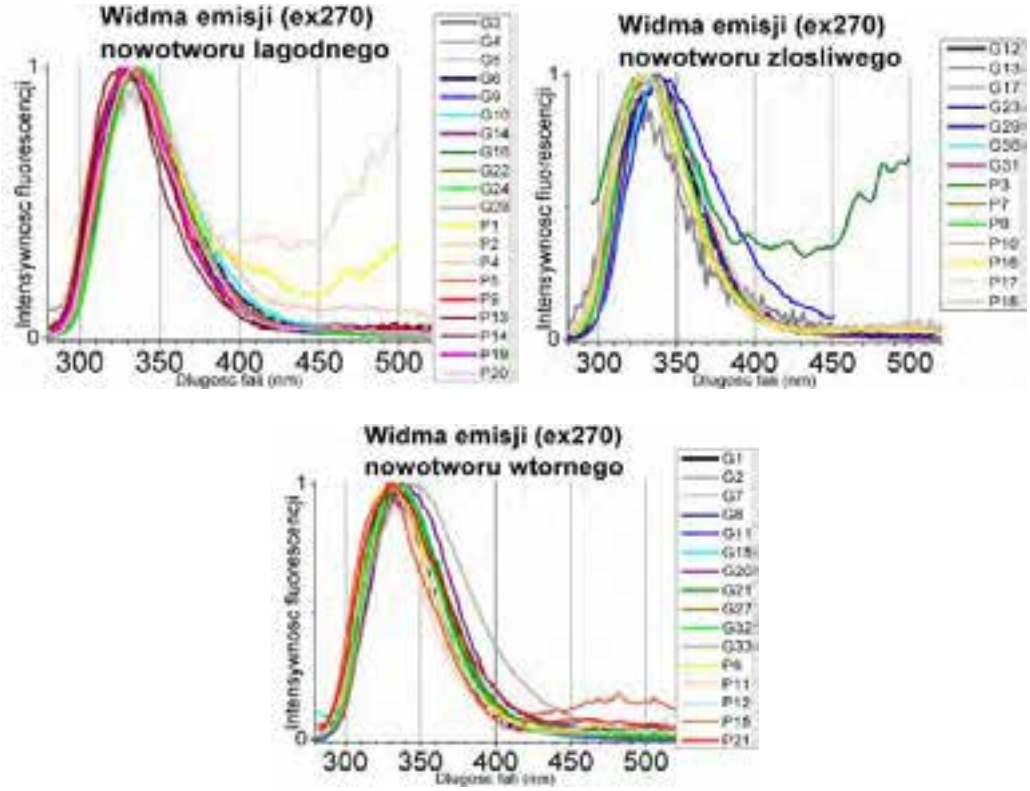

Figure 2. Normalized emission spectra at 270 nm excitation of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastatic tumors

EMISSION SPECTRUM WHEN EXCITED WITH 290 NM LIGHT OF CNS TUMORS

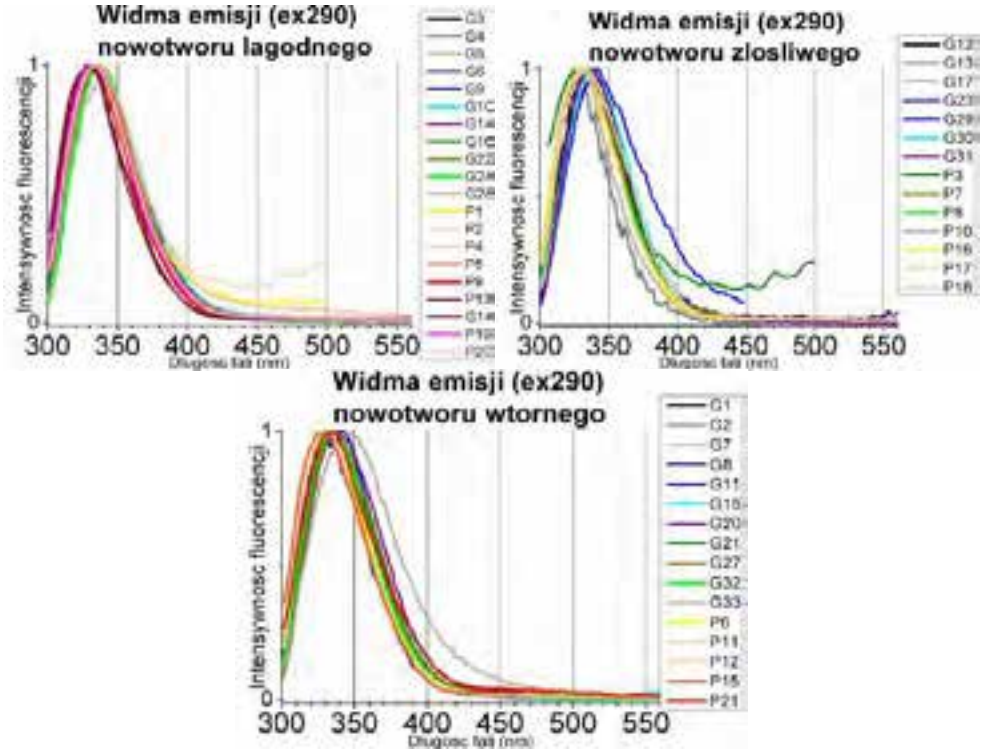

Figure 3. Normalized emission spectra at 290 nm induction of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastatic tumors

EMISSION SPECTRUM WHEN EXCITED WITH 310 NM LIGHT OF CNS TUMORS

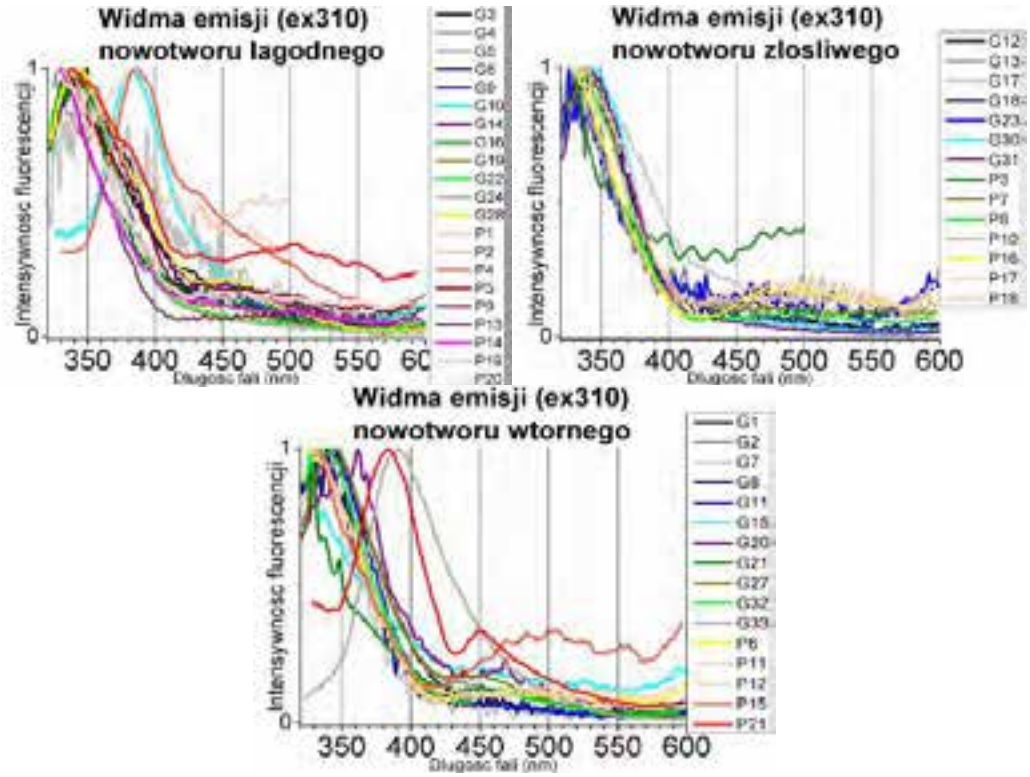

Figure 4. Normalized emission spectra at 310 nm induction of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastatic tumors

EMISSION SPECTRUM WHEN EXCITED WITH 330 NM LIGHT OF CNS TUMORS

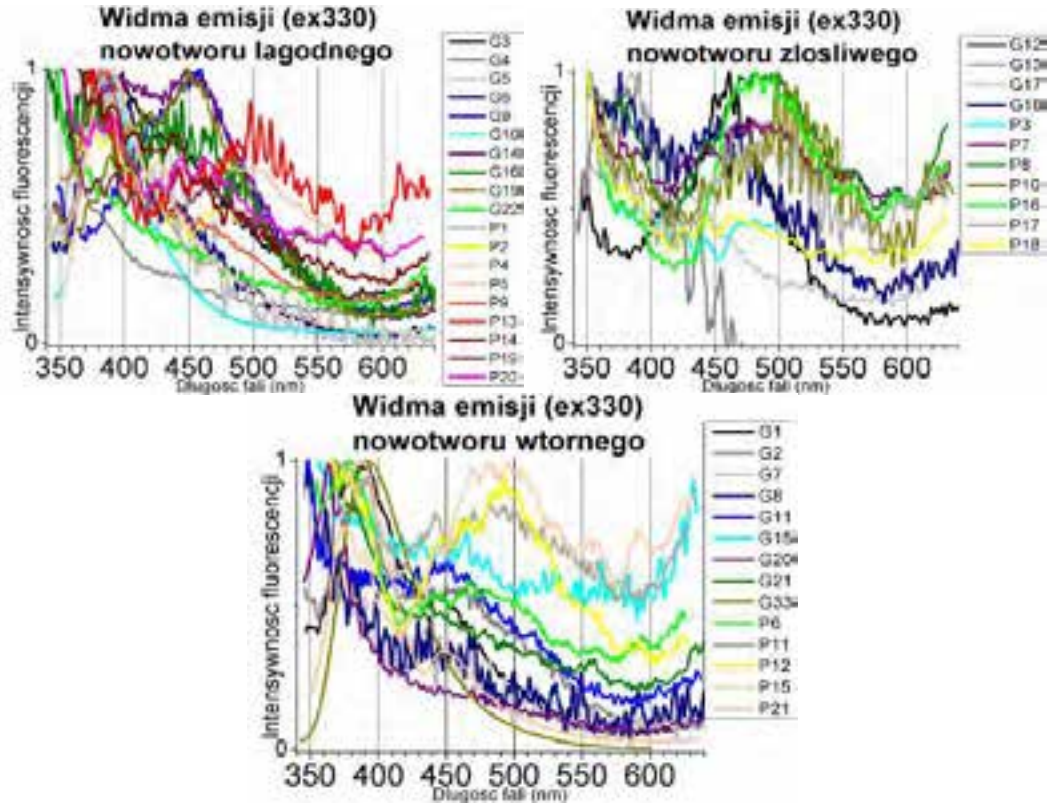

Figure 5. Normalized emission spectra at 330 nm induction of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastatic tumors

EMISSION SPECTRUM WHEN EXCITED WITH 350 NM LIGHT OF CNS TUMORS

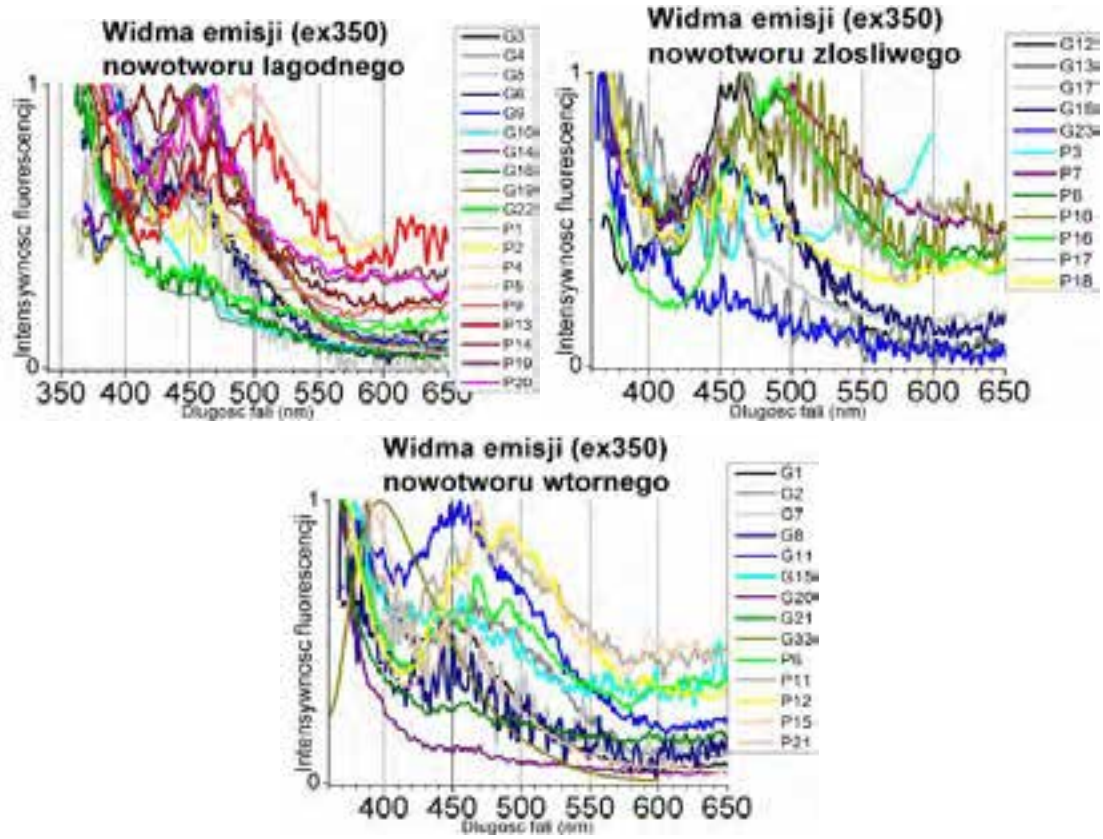

Figure 6. Normalized emission spectra at 350 nm induction of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastatic tumors

EMISSION SPECTRUM WHEN EXCITED WITH 370 NM LIGHT OF CNS TUMORS

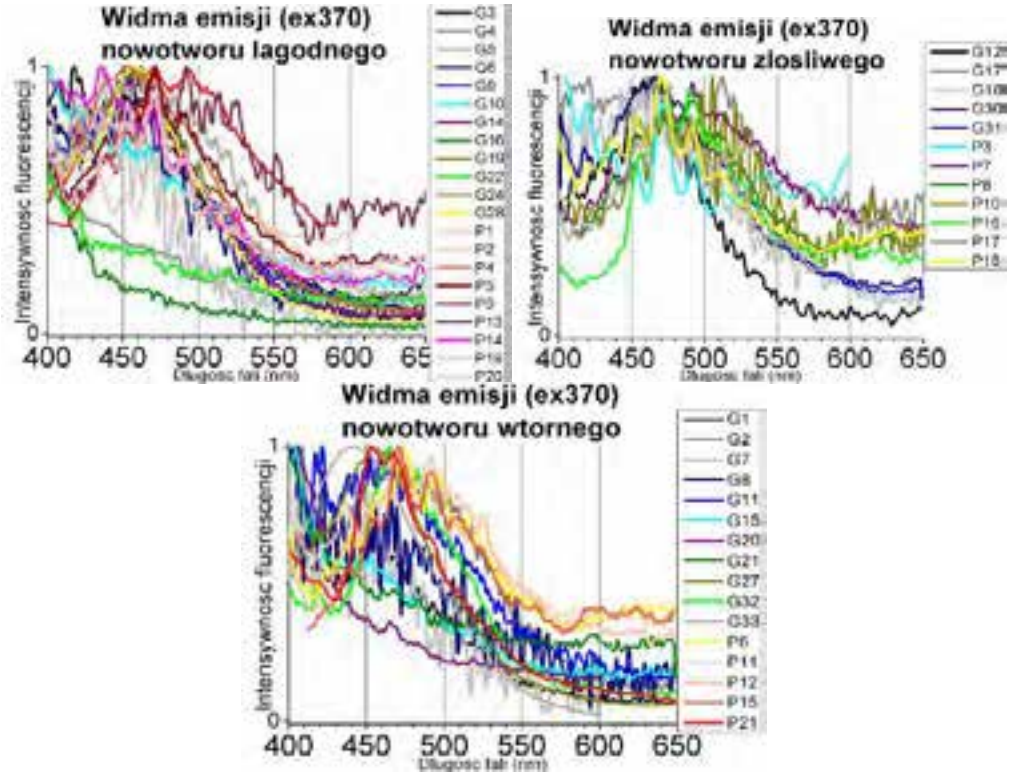

Figure 7. Normalized emission spectra at 370 nm induction of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastatic tumors

EMISSION SPECTRUM WHEN EXCITED WITH 390 NM LIGHT OF CNS TUMORS

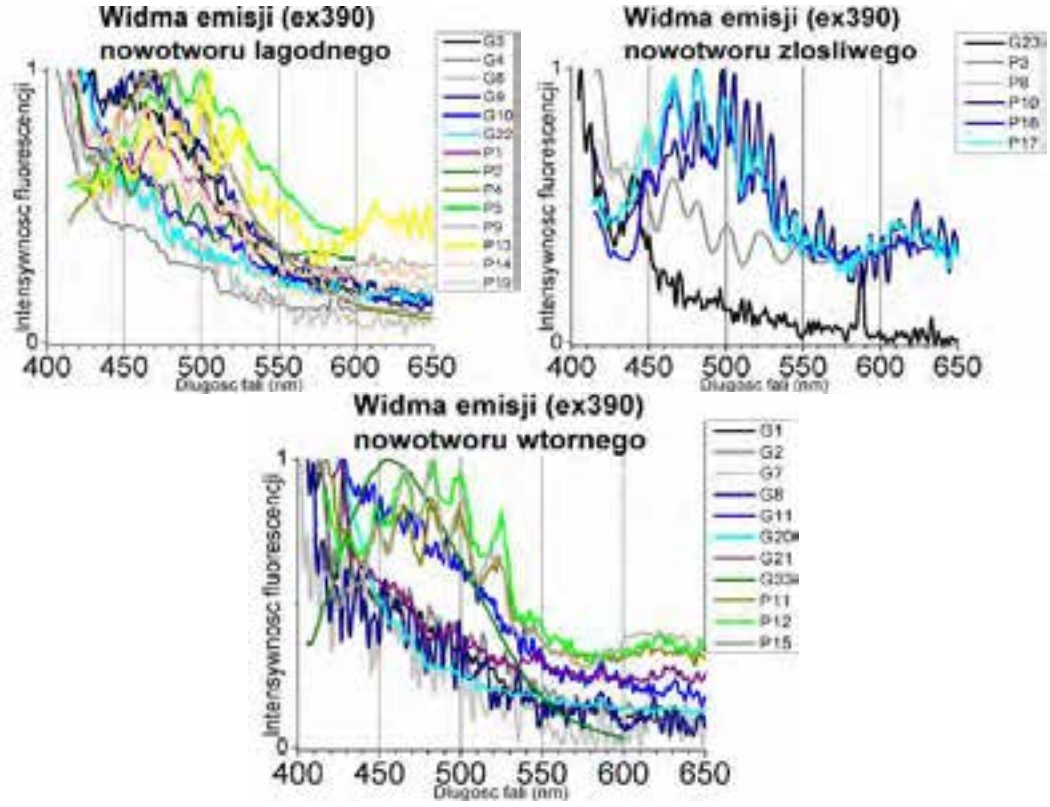

Figure 8. Normalized emission spectra at 390 nm induction of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastatic tumors

Fluorescence emission spectra recorded at 250 nm light excitation are shown in figure 1. For all CNS tumor types, two major maxima can be distinguished – at 335 nm and 390 nm.

In contrast, in figure 2, showing emission spectra for excitation with 270 nm light, we only see one maximum at 335 nm.

Similar results can be seen in figure 3 showing the emission spectrum when excited with 290 nm light.

All results of the Pearson correlation analysis were at the p-level close to zero ($p < 0.05$), i.e. all results are statistically significant at $\alpha = 0.05$.

The emission spectra were statistically analyzed at excitation with 290 nm (ex290). The length of ex290 was chosen because from the correlation analysis it can be concluded that there are large differences between benign and malignant tumor and between malignant and secondary cancer. Such a combination would allow to show differences between all types of tumors (see fig. 3)

For the statistical analysis of the emission spectra at excitation, the length at which the band reaches the maximum fluorescence was chosen because the spectra are characterized by a single maximum – in relation to which the maxima ratio can not be compared. The lengths at maximum fluorescence, broken down by tumor group, are listed in the table (tab. 1).

Table 1. Table containing list of length at maximum fluorescence for emission spectra at excitation 290 nm with division into tumor groups: 1 – secondary cancer, 2 – malignant tumor, 3 – benign tumor

Patient	ex290 max	
	group	length at max fluorescence
G1	1	335,6
G2	1	335,2
G7	1	333,8

G8	1	330,8
G11	1	342,8
G15	1	338,2
G20	1	336,4
G21	1	335,6
G27	1	336,6
G32	1	336,4
G33	1	348
P6	1	331,6
P11	1	331,4
P12	1	329,6
P15	1	330,2
P21	1	336,6
G12	2	333,6
G13	2	323,8
G17	2	337,4
G23	2	332,6
G29	2	342,2
G30	2	337,6
G31	2	339,4
P3	2	328
P7	2	334,8
P8	2	333,2
P10	2	329,4
P16	2	328
P17	2	331,2
P18	2	330,2
G3	3	331
G4	3	328,2
G5	3	339,4
G6	3	334
G9	3	337,6
G10	3	338,4
G14	3	337,8
G16	3	334,4

G22	3	337,8
G24	3	335,2
G28	3	334,6
P1	3	331,2
P2	3	330,8
P4	3	338
P5	3	333,8
P9	3	332,2
P13	3	332,6
P14	3	327,2
P19	3	334,4
P20	3	332,8

Next, the consistency of the distributions in the three groups with the normal distribution were compared (tab. 2, 3, 4). It was assumed that $\alpha = 0.05$ and for assumed hypotheses H0 – the distribution is with normal distribution and H1 – the distribution is not a normal distribution

Table 2. Compatibility of distribution in group 1 – secondary cancer (metastasis)

Variable	group = 1(ex290 max)		
	N	W	P
length at max fluorescence	16	0,886237	0,048586

Table 3. Compatibility of the distribution in group 2 – malignant tumor

Variable	group = 1 (ex290 max)		
	N	W	P
length at max fluorescence	16	0,886237	0,048586

Table 4. Compatibility of distribution in group 3 – benign tumor

Variable	group = 3 (ex290 max)		
	N	W	P
length at max fluorescence	20	0,956355	0,473848

STATISTICAL ANALYSIS OF MAXIMUM SPECTRUM FLUORESCENCE

Only for the first group – neoplastic secondary distribution is not a normal distribution, because $p < 0.05$. Therefore, the nonparametric Kruskal-Wallis test was chosen for further analysis. For the test, the significance level $\alpha = 0.05$ and H0 were assumed – the distributions are the same, and the H1-distributions differ. The results are shown in the table below (tab. 5).

Table 5. Kruskal-Wallis nonparametric test result table for the length at which the maximum spectrum fluorescence is found for the groups: 1 – secondary cancer, 2 – malignant tumor, 3 – benign tumor

	ANOVA rang Kruskala-Wallisa; length at max fluorescence (ex290 max) Independent (grouping) variable: group Test Kruskala-Wallisa: H (2, N = 50) = 1,984965 p = 0,3707			
Dependent: · length at max fluorescence	Kod	N ważnych	Suma Rang	Średnia Ranga
1	1	16	461,0000	28,81250
2	2	14	298,5000	21,32143
3	3	20	515,5000	25,77500

The critical value of the Kruskal-Wallis test for two degrees of freedom is 5,991,464. The result of the Kruskal-Wallis test H = 1.984965 and p = 0.3707 show that the groups do not differ statistically with each other because the test result H < Critical value and p > 0.05.

DISCUSSION

Investigations of autofluorescence spectroscopy are a method that exploits the ability of endogenous fluorophores to fluoresce light of a different length than an excitation light. There are numerous molecules capable of autofluorescence in the tissues of living organisms.

In addition to the maximum at a length of about 335 nm, we observe for some cases (regardless of the type of cancer), an additional band with a maximum at 450 nm and/or 500 nm. It is intriguing that for cases G10, P2 and P4 with benign tumor and G33 and P21 with secondary cancer (metastasis), there was a shift in the maximum from 335 nm to about 385 nm. In addition, one can observe a certain tendency (although this is not the rule) – this applies to the second band, where the maximum is at 450 nm and/or 500 nm. For cases with benign tumors, most spectra have a maximum at 450 nm, and with malignant tumors, most spectra have a maximum at 500 nm. In graph 5 a shift in the maximum (for most spectra) from 335 nm to 385 nm was observed. In addition, an increase in fluorescence was recorded within the 450-550 nm band in relation to the maximum at 385 nm. Also here, there was a tendency to increase the maximum at 450 nm for benign tumors, and the maximum at 500 nm is more characteristic of malignant tumors.

Secondary tumors (metastases) are a mixture of spectra with a predominance of maxima at 450 nm or 500 nm. Unfortunately, also in this case it is not a rule. The samples marked as G10, P2 and P4 with benign tumor as well as G33 and P21 with secondary cancer still diverge from the others. When fluorescence was excited with 350 nm light in the 6,370 nm graph on the 7 and 390 nm graphs in Figure 8, the same trends were also observed – the maxima are the same as for the spectra shown on Figure 5. For benign malignancy tumors maxima are observed at the 450 nm, for malignant tumors at 500 nm, and for secondary cancers between 450 nm and 500 nm.

In the conducted stationary studies, numerous fluorescence spectra were obtained, indicating the presence of endogenous chromophores in the analyzed biological material. By excitation with light lengths of 250-290 nm, the amino acids contained in proteins are primarily induced. They are phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp). They emit fluorescence in the range of 250-450 nm with maxima found at 282 nm, 303 nm and 348 nm (Richards-Kortum, 1996). Therefore, it is likely that the spectra of light excitation excited at 250 nm, 270 nm, 290 nm and 310 nm show a band with a maximum of 330-340 nm corresponding to the fluorescence of amino acids (above all to tryptophan). The spectra also show a band with a maximum of about 390 nm. According to the data presented, it may be pyridoxine or/and collagen (Skoch, 2008). The fluorescence of these chromophores is probably excited by 330 nm light, as can be seen in graph 13 showing the excitation spectra at 390 nm light emission. The next significant maxima in the emission spectra are in the 450 nm and 500 nm positions. According to the data for fluorescence with a wavelength of 450 nm, nicotinamide, NADPH, which are excited by successive light with a length of 360 nm and 366 nm, may be responsible (Trehin, 2006). 330-340 nm. Fluo-

rescences with a length of 500 nm are observed for elastin, which has maxima of excitations 350 nm, 410 nm and 450 nm (Senada, 2012; Teale, 1956; Pu, 2012).

The stationary spectra of individual cases differed among themselves, which suggests a just attempt to use this method for diagnostic purposes. It was suggested to divide the examined material into benign, malignant and metastatic CNS tumors. In the studied groups, there were often cases whose spectra differed significantly from the others. Unfortunately, the spectra obtained in each group were not completely consistent. Large differences in the shape of spectra within individual groups have been shown. These discrepancies may be due to the fact that in addition to the type of cancer, other factors such as medicines used, the exact location of biological material, and co-morbidity may also affect tissue fluorescence. Therefore, to make further divisions, which in a more systematic way could indicate differences in fluorescence between groups, it is necessary to increase the number of samples tested to be able to correctly distribute cases.

Researchers from China, Yan Zhou (Zhou, 2012), studied the brain and brain tumors unharmed. They had at their disposal three different types of tissues: normal, malignant tumor, benign tumor. Using fluorescence excitation of 300 nm, they distinguished malignant tumor from benign tumor and healthy tissue. Tissues covered by benign or malignant tumor lesions are characterized by a band with one maximum at a length of about 340 nm and a small fluorescence in the 400-550 nm range. These data correspond perfectly with those obtained in this work.

The obtained test results and their verification with the results of other scientists dealing with fluorescence spectroscopy allow to confirm the main assumption of this study that the assessment of excitation spectra, emission spectra and fluorescence decay time in patients with central nervous system tumors is of great clinical significance in determining prognosis, planning and monitoring the activity of these cancers (Fillipi, 2001; Geiger, 2011; Hayashida, 2006). Planning and appropriate treatment selection in case of intracranial tumors is very complex and often determines further prognosis. The photodynamic method is distinguished by the relatively low invasiveness of the surgery itself and, at the same time, by high sensitivity and resolution in comparison to traditional diagnostic methods such as: nuclear magnetic resonance, computed tomography, ultrasound (Hongwei, 2014; Petrovsky, 2003; Toms, 2005). Hence, spectrophotometric analysis of brain tumors may be extremely useful. This will allow to make even more accurate diagnosis and to start the appropriate therapy (Yaroslavsky, 2002; Yinghua, 2010; Bergner, 2012).

CONCLUSIONS

Not without significance is the fact that the use of spectroscopic methods will allow very fast analysis of the material collected. The physician performing the examination will be informed in a short time whether the material collected by him shows the spectral characteristics of the changed cells and whether the material should be taken again from another place. The results of the study, limited by the small size of the group, do not allow for binding conclusions, but they confirm the usefulness of the method in neuro-oncological cases. Fluorescent techniques will not displace pathomorphological analysis in the near future.

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New trends in glioblastoma multiforme immunotherapy

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ABSTRACT

Glioblastoma multiforme is a malignant brain tumour characterized by extremely high mortality. For this reason, the attention of many scientists is focused on developing innovative therapies. Recent studies provide satisfactory data on the effectiveness of immunotherapy in some solid tumours, which raises hope also for glioblastoma treatment.

The aim of this review is to present the current state of knowledge in the field of glioblastoma immunotherapy.

The goal of immunotherapy is to change the immunosuppressive microenvironment of the tumour and to mobilize the patient's immune system, which leads to the eradication of tumour cells. One of the methods studied with glioblastoma is the use of cancer vaccines. It is based on direct exposure to antigens or stimulation of the patient's antigen-presenting cells. Another method that may prove effective is the use of oncolytic viruses. The positive effect of the therapy is not only due to the destruction of the cancer cell itself, but above all is the result of the release of antigens from it which mobilize the immune system. Another approach to overcoming tumour immunosuppression is the use of immune checkpoint inhibitors which have been shown to be effective in other cancers. CAR-T cell therapy may also be a new potential treatment for gliobla-stoma multiforme. It is based on genetic modification of T cells taken from the patient to produce glioma-specific surface antigens, which allows interaction with tumour cells leading to cell death. Apart from the use of monotherapy, the use of combined treatment methods is also of great interest. The possibilities of immunotherapy in glioblastoma are very limited, mainly due to its immunosuppressive microenvironment and the presence of multiple mechanisms of therapy resistance.

Undoubtedly, research on immunotherapy of glioblastoma contributes to the development of new treatment regimens that may prove most effective in the case of a therapy combined with other treatments.

INTRODUCTION

Glioblastoma multiforme (GBM) is rare and one of the most lethal among malignant solid tumours. The incidence of this disease increases rapidly after the age of 54 years, reaching a median of 64 years (Alexander, 2017). Current standard therapy consists of maximal tumour resection, radiotherapy and chemotherapy with temozolomide followed by adjuvant temozolomide therapy (Alexander, 2017; Chen, 2018). Unfortunately, among patients diagnosed with the disease, the average survival time is less than 15 months even with standard therapies (Chen, 2018). Given this grim prognosis, researchers around the world are attempting to develop effective therapies.

Early treatment failure has been attributed to many factors. The microenvironment of this cancer is incredibly diverse and also varies between patients (DeCordova, 2020). It also contains many non-cancerous cells, most notably immune cells. Much attention has been paid to tumour-associated macrophages which are associated with tumour progression, among other things (Chen, 2018). In the case of glioblastoma multiforme, a nonimmunogenic "cold" environment is observed (Young, 2020). This is associated with many features of the tumour, such as decreased expression of MHC class I molecules, impaired antigen presentation by APC cells, or an increase in immunosuppressive cells (Saha, 2018). All of these features prevent the recognition of the tumour by the immune system and, consequently, the body's effective fight against cancer. Moreover, tumours escaping immune surveillance cause the immune system to not only fail to fight the tumour, but may also act in its favour (Chen, 2018).

Immunotherapy is an innovative treatment method that involves modulating the immune system in a way that allows it to attack cancer cells. The regulation of the immune environment resulting from such therapy has been shown to be effective in the treatment of several cancers. Combining immunotherapy with other treatments to enhance its effect seems particularly promising (Tan, 2020). Numerous data on the effectiveness of immunotherapy in cancer treatment provide hope for patients with glioblastoma multiforme. Despite numerous clinical trials, to date no immunotherapy has been approved by the Food and Drug Administration (FDA) for the treatment of glioblastoma multiforme (McGranahan, 2019). There are several factors

that undoubtedly contribute to the low efficacy of this type of therapy. The first problem is the physical blood-brain and blood-tumour-brain barrier, which is an obstacle to therapeutic molecules (Young, 2020). Another problem is the effects of therapies usually used in glioblastoma, namely temozolomide and radiation, which lead to lymphopenia. Glioblastoma patients are also given dexamethasone, a synthetic corticosteroid which relieves brain swelling but also leads to suppression of the immune system (McGranahan, 2019; Young, 2020). Immunosuppression within the tumour, mentioned earlier, and its high resistance to treatment resulting from, among other things, the high heterogeneity of the tumour, as well as its low tumour mutational burden, are also not without significance (Medi-konda, 2021).

The aim of this paper is to review recent studies on immunotherapy for glioblastoma multiforme, both as monotherapy but also in combination with other treatments.

SEARCH STRATEGY AND SELECTION CRITERIA

The following article was compiled from both review and original scientific publications, including data obtained from clinical trials. The PubMed database was used primarily to search for sources. The entering terms was: glioblastoma multiforme immunotherapy, glioblastoma therapy, CAR-T glioblastoma, glioblastoma vaccines, glioblastoma virotherapy, oncolytic viruses glioblastoma, immune checkpoint inhibitors glioblastoma and combined therapy glioblastoma. The cited articles were published between 2010 and 2021 and, except for one, are all in English. The ClinicalTrials.gov database was also used in compiling this review.

REVIEW

CAR-T THERAPY FOR THE TREATMENT OF GLIOBLASTOMA MULTIFORME

With the progress and development of genetic engineering, it has become possible to model and reprogram cells, including the patient's own immune cells, to fight a specific disease entity. Such therapy has been called adoptive immunotherapy. Chief among such forms of therapy is CAR-T therapy, or Chimeric Antigen Receptor T cells whose appropriately engineered receptors are capable of interacting with antigens of cancer cells, including potentially glioblastoma multiforme (Marei, 2021). The process of immunotherapy with the use of genetically modified CAR-T cells takes place in seve-ral stages starting from the collection of leukocytes from the patient's blood through their genetic modification with the use of viral vectors, and then administering them back to the patient's body from which they were isolated (Giotta Lucifero, 2021). This method is currently approved by the FDA for the treatment of mainly B-cell lymphomas and leukaemias in paediatric patients, but the potential of this therapy is also being explored for other cancers, including GBM (Marei, 2021).

The structure of CAR molecules varies and, due to the number of costimulatory domains of the CAR receptor, a division into five generations has been made. Generation I contains a fragment of the CD3ζ chain, while II and III contain, in addition to the fixed CD3ζ fragment, successively one or two additional costimulatory domains, e.g. CD22, OX40 or 4- 1BB (Choi, 2019; Marei, 2021). In generation IV a cytokine domain is additionally present and in generation V, a cytokine receptor-binding domain is present (Feldman, 2021; Sterner, 2021; Yu, 2021). The overall structure of the CAR molecule consists of several elements, these are: the extracellular domain, whose function is to recognise and interact with the relevant antigen of tumour cells, connected by a hinge region to the second element, i.e. the endothelial domain which in turn is connected to several T-cell-derived intracellular chains. The function of the intracellular domain is to induce the T cell pathway. The only variable element in the structure of the CAR molecule is the antigen recognition domain, the appropriate selection of which influences the proper interactions with tumour cells (Choi, 2021; Giotta Lucifero, 2021; Marei, 2021; Sterner, 2021; Yu, 2021).

In clinical trials for the potential use of CAR-T therapy in the treatment of glioblastoma multiforme, specific receptors that are overexpressed in the majority of GBM patients have received much attention, these include, e.g. Interleukin-13 Receptor A2 (IL-13Rα2), Human Epidermal Growth Factor Receptor 2 (HER2) and Epidermal Growth Factor Receptor variant III (EGFRvIII). As most of these are activated in the tumourigenic process they are excellent

molecular targets for CAR-T treatment of GBM (Giotta Lucifero, 2021; Marei, 2021).

One of the most important receptors, with tyrosine kinase activity, associated with this type of cancer is HER2 (Feldman, 2021). This factor is responsible for cell proliferation, adhesion and survival (Feldman, 2021). It is expressed in both healthy tissues and in approximately 80% of cases in GBM, therefore targeting this receptor can induce an immune response against healthy body tissues, as observed in a 2010 study (Feldman, 2021; Morgan, 2010). Accordingly, in 2017, Ahmed et al. (Ahmed, 2017) conducted a study on a group of 17 individuals using CAR-T therapy containing a CD28 costimulatory domain and an extracellular domain directed against the HER2 receptor in GBM with overexpression of this receptor. The results of this study showed that the use of infusions of CAR-T cells with an anti-HER2 domain could induce a partial tumour response within 6 months of administration. In addition, CAR-T cells were shown to persist in the body for 12 months with no toxicity, which carries much hope for the future safe use of CAR-T in GBM therapy (Giotta Lucifero, 2021; Marei, 2021; Sterner, 2021).

The IL13Rα2 receptor, which is expressed only in tumour cells, is also promising for the treatment of glioblastoma multiforme (Marei, 2021). This factor is a major prognostic indicator and is associated with poor prognosis and the possibility of metastasis and tumour growth through activation of the PI3K/AKT/mTOR pathway (Feldman, 2021). It represents an accomplished molecular target as its expression is 75% correlated with glioblastoma multiforme. To date, two studies have been conducted using CAR-T cells directed against the IL13Rα2 antigen. The first study from 2015 by Brown et al. focused on the administration of first-generation CAR cells with an anti-IL13Rα2 extracellular domain to three patients by intravenous infusion. On completion of the CAR-T cell therapy, they observed significantly reduced IL13Rα2 expression in tumour cells, which contributed to the expansion of the study to include the use of second-generation CAR cells with a 4-1BB costimulatory domain. The results of the 2016 study thus demonstrated that this treatment method using higher-generation CAR cells allows local tumour cell death at the site of administration. In addition, the increased induction of an immune response as well as the safety of such therapy has been proven (Choi, 2019; Feldman, 2021; Giotta Lucifero, 2021; Marei, 2021).

In the case of cancer-related diseases, frequently depending on the location of the tumour, antigens that are specific to a particular type of cancer can be identified. In the case of glioblastoma multiforme, one such antigen is a mutated form of EGFRvIII (Choi, 2019). The mutation observed here relative to wild-type EGFR is the deletion of exons 2-7, resulting in the translocation of a glycine residue in the extracellular domain, making it impossible for the receptor to bind to its ligand. The hallmark of this form is its specific expression in glioblastoma multiforme cells and relatively low expression in normal body cells (Choi, 2019; Feldman, 2021). In 2017, O'Rourke et al. conducted a study on a group of 7 patients with glioblastoma multiforme with EGFRvIII over-expression diagnosed and confirmed by next-generation sequencing. Second-generation modified CAR cells containing a CDζ domain and a 4-1BB costimulatory domain along with an anti-EGFRvIII extracellular domain were used for treatment. CAR-T cells were administered by intravenous infusion. Following therapy, patients underwent tumour surgery to assess the therapeutic potential. The study found significantly reduced levels of mutant EGFRvIII receptors within the tumour tissue and increased levels of molecules responsible for immunosuppressive processes, i.e. PD-L1, IL-10 and Transforming Growth Factor β (TGF-β) (Choi, 2019; Feldman, 2021; Giotta Lucifero, 2021). Further clinical studies on the use of EGFRvIII-targeted CAR-T cells in GBM are currently underway.

With CAR-T therapy, it is possible to conduct a targeted immune response by selecting the appropriate external domain depending on the disease entity. It should be noted that the majority of studies have shown no toxicity associated with the administration of CAR-T cells to the human body, which indicates that this therapy may prove effective in the treatment of other diseases besides cancer. Clinical trials are currently being conducted on CAR-T therapies targeting other antigens associated with GBM, such as B7-H3, CD147 or MMP2 (Maggs, 2021).

CANCER VACCINES

One type of immunotherapy of great interest to brain tumour researchers is cancer vaccines. It is postulated that such vaccines would promote an anti-tumour involving the adaptive immune system (Medikonda, 2021). If we talk about a vaccine in the context of cancer therapy, it should be noted that its aim is to generate immunity against tumour antigens, which should ultimately lead to the destruction of tumour cells (Cuoco, 2018). The aim of this action is therefore not to prevent the disease, as in the case of vaccines against infectious diseases, but to lead to the eradication of tumour cells (McGranahan, 2019). For the treatment of glioblastoma, a distinction is made between peptide or DNA vaccines, which result in direct exposure to tumour antigens. These antigens are used together with immune response stimulators. For glioblastoma, there are few tumour-specific antigens, so common tumour-associated antigens are often used in vaccines (McGranahan, 2019). Another type of vaccines are those "tailor-made" for individual patients, which are created by stimulating dendritic cells previously collected from the patient (McGranahan, 2019).

As previously mentioned for glioblastoma, not many tumour-specific antigens are known, but one of them is the EGFRvIII variant, a constitutively activated epidermal growth factor mutation found in about 25-30% of patients (McGranahan, 2019; Medikonda, 2021). The use of EGFRvIII as a vaccine antigen results in its low toxicity, as this variant is not expressed outside the tumour environment. However, on the other hand, the fact that it is only present in the tumours of some patients makes this therapy likely to be effective only in a selected, limited group of patients (Medikonda, 2021). Furthermore, in the case of tumour-specific antigens, their uneven expression inside the tumour is observed, which, if treated, results in the death of only a part of the tumour cells. This problem is even more serious as tumour cells may be able to promote the growth of cells not expressing a given antigen, which contributes to disease recur-rence (Wilcox, 2018). An example of EGFRvIII antigen-based therapy is rindopepimut, a vaccine consisting of a 14 amino acid peptide combined with the immunogenic carrier protein keyhole limpet hemocyanin (KLH) (Wilcox, 2018). Patients undergoing total resection and chemotherapy were eligible for phase II clinical trials and an increase in median overall survival (mOS) to 24 months was noted (Medikonda, 2021). Unfortunately, despite the promising results of phase II clinical trials, phase III trials were suspended because they did not yield the expected results (Daubon, 2020; Medikonda, 2021; Wilcox, 2018). In contrast, the results of a phase II study of the efficacy of the combination of rindopepimut with bevacizumab, in which an increase in patients' mOS was observed, appear promising (McGranahan, 2019; Medikonda, 2021; Muir, 2020). It is clear that rindopepimut has some pharmacological activity against GBM in a specific group of patients, but further studies are needed to develop specific treatment regimens, including but not limited to the addition of anti-angiogenic therapy and patient selection.

The low number of tumour-specific antigens creates the need to develop vaccines based on tumour-associated antigens. An example of such a vaccine is SurVaxM containing a survivin mimic peptide conjugated to KLH (McGranahan, 2019). Survivin belongs to the family of inhibitors of apoptosis and its overexpression is observed in many types of cancer, including GBM (Cuoco, 2018; Winograd, 2016). Furthermore, its levels correlate with disease progression (Cuoco, 2018). Studies of this vaccine have shown no serious side effects, and patients have been observed to develop humoral and cell-mediated immune responses. SurVaxM is currently being studied in combination with temozolomide treatment (Cuoco, 2018).

Another approach uses multipeptide vaccines which reduces the risk of immune tolerance and recurrence (Cuoco, 2018). An example is the IMA950 vaccine containing multiple tumour-associated peptides that are present in glioblastoma tissues. This includes the survivin discussed earlier, but also other antigens such as brevican (BCAN); chondroitin sulfate proteoglycan 4 (CSPG4); fatty acid-binding protein 7 (FABP7); IGF-2 mRNA-binding protein 3 (IGF2BP3); neuroligin 4, X-linked (NLGN4X); neuronal cell adhesion molecule (NRCAM); protein tyrosine phosphatase, receptor-type, Z polypeptide 1 (PTPRZ1); tenascin C (TNC); Met protooncogene (MET) (Cuoco, 2018; Winograd, 2016). Phase I trials of this vaccine provided satisfactory results, with 90% of patients observed to produce a single immune response, and 50% observed to respond to multiple, or at least two, antigens (Cuoco, 2018; Winograd, 2016).

Another multipeptide vaccine being tested in glioma is SL701. It is based on short synthetic peptides targeting IL-13Rα2, ephrin A2 and survivin. The study observed a CD8 response, which was associated with prolonged patient survival (McGranahan, 2019). Analysis of SL701 in combination with bevacizumab showed that the vaccine is well tolerated and has anti-tumour activity (Cuoco, 2018).

Vaccines using dendritic cells (DC) are also of interest among researchers. Dendritic cells, which are involved in the natural anti-tumour response, are stimulated with anti-gens from the patient's tumour and then delivered to the patient (Desland, 2020). Such vaccines can be derived using synthetic or tumour-derived peptides, RNA or crude tumour lysate. DC cell-based vaccines have advantages over cell-free peptide vaccines in that they can contain a much larger number of antigens (Winograd, 2016). Results from several preclinical studies suggest that such therapies have potential in the treatment of glioblastoma, as they increase CD8+ T-cell infiltration deep into the tumour, which is associated with increased survival (Desland, 2020). In the case of GBM, the production of individual vaccines for patients is possible if the tumour is located such that surgical intervention is possible, as vaccine production requires taking a section of the tumour. This unfortunately limits the group of patients for whom production of such a vaccine is likely (McGranahan, 2019). Clinical trials of DC vaccines have provided data supporting their excellent safety profile. In addition, they have also demonstrated mobilisation of the immune system of patients and initiation of humoral and cellular immunity to some extent (Desland, 2020). Among the DC vaccines that have been evaluated in clinical trials, the DC-Vax-L deserves special attention. It is produced by pulsing autologous patient dendritic cells using whole tumour lysate. It seems highly promising that some patients who received DC-Vax-L in phase I and phase III clinical trials have a survival of more than 10 and 7 years after administration, correspondingly (McGranahan, 2019).

Personalised peptide vaccines may also be the future of therapy in GBM. The "Glioma Actively Personalised Vaccine Consortium" (GAPVAC) study evaluated the impact of treatment with two personalised vaccines with co-administration of temozolomide and radiotherapy (Dunn, 2020). The antigenic composition of the first vaccine was based on expression, physical HLA binding and immunogenicity, resulting in a vaccine composed of seven antigens. Subsequently, patients received a second vaccine containing two neoantigenic peptides that were identified by sequencing and HLA binding affinity (Dunn, 2020). In 11 of the 15 patients tested, both CD8+ and CD4+ cell responses were observed in reaction to these two vaccines. Moreover, the vaccines were well tolerated. The median progression-free survival and overall survival of 14.5 and 29.0 months, respectively, seems extremely optimistic. (Dunn, 2020). The results of these studies are encouraging and provide hope that the future of glioblastoma multiforme treatment may rely heavily on personalisation of therapy.

The use of vaccination in the treatment of glioblastoma multiforme may prove effective in the future. The data on increasing mOS are very promising. Unfortunately, more research is needed and, perhaps, to tailor the vaccines being tested to the molecular subtype of glioblastoma multiforme.

VIROTHERAPY

Interest in viruses as agents for anticancer therapy dates back over a century ago. Years of research in oncology and virology have led to the identification of two types of anticancer virotherapy. The first, using replication-competent viruses that are oncolytic, i.e. capable of infecting a cancer cell, which then undergoes lysis and releases viral progeny capable of infecting subsequent cancer cells (Foreman, 2017). Initially, it was thought that their positive impact lay solely in the destruction of cancer cells, but this is only one side of the coin. Oncolytic viruses exert an anti-tumour effect primarily because when they damage a cell, they cause the release of tumour-associated antigens and damage associated molecular patterns from the cell which are then recognised by the body and mobilise the immune system to fight the tumour (Desland, 2020; Fecci, 2019; Martikainen, 2019). In addition, oncolytic viruses themselves contain pathogen associated molecular patterns, the release of which is also associated with tumour cell destruction, as these patterns are recognised by the innate immune system (Fecci, 2019). Certain features of glioblastoma multiforme favour the use of oncolytic

virus therapy for its treatment. This is related, among other things, to the fact that glioblastomas do not give rise to distant metastases, so the disease is restricted to one organ only. Moreover, these tumours grow surrounded by nondividing cells, which favours the retention of oncolytic viruses in a restricted area, as they multiply exclusively in dividing cells (Wollmann, 2012). The second type of anticancer virotherapy relies on viruses incapable of replication as a vector to deliver therapeutic genes (Foreman, 2017). An example of the use of virotherapy in the treatment of GBM is the use of modified herpes simplex viruses with an embedded transgene encoding interleukin-12, a cytokine whose anticancer activity is inter alia due to its ability to induce interferon gamma (IFNγ) expression (Nguyen, 2020).

Several clinical trials are evaluating the effect of oncolytic viruses in the treatment of glioblastoma multiforme. Preliminary results from clinical trials of adenoviruses, herpes simplex virus, and replicating retroviruses have shown increased patient survival (Khansur, 2019). In addition, other viruses such as polio virus, cowpox virus and measles virus, among others, are also being studied. Of particular interest are the results of studies on DNX-2401, PVS-RIPO and Toca 511, against which an immune response was observed in 20% of GBM patients (Martikainen, 2019).

DNX-2401 is a viral vector based on oncolytic adenovirus 5. This virus has been modified to increase infectivity, it replicates in GBM cells and is specific for this cancer (Stepanenko, 2018). Specificity towards GBM results from the introduction of two rearrangements: modification of the E1A gene and addition of an arginine-glycine-aspartic acid motif. It is also associated with restriction of its replication in normal cells (Philbrick, 2019). Among patients to whom the virus was administered either by direct single injection into the tumour or using a catheter, survival of more than 3 years was observed in 20% of the patients studied. Furthermore, survival beyond 3 years with progression-free survival after treatment was observed in three patients (Stepanenko, 2018). DNX-2401 is also being studied in combination with other therapies, including temozolomide or pembrolizumab. It is worth mentioning that the therapeutic effect of DNX-2401 is due to both its ability to destroy tumour cells and generate an anti-tumour immune response (Philbrick, 2019).

PVS-RIPO is a live attenuated polio virus type 1 (Sabin) vaccine containing heterologous IRES from human rhinovirus type 2 (Walton, 2018). Virus entry into the cell occurs through interaction with the CD155 receptor, which is upregulated on the cell surface of solid tumours and in the tumour microenvironment. Moreover, this receptor is also expressed on the surface of antigen-presenting cells (Desjardins, 2018; Fecci, 2019). *In vitro*, this virus induces neoplastic cell death and promotes the release of proinflammatory cytokines (Desjardins, 2018; Fecci, 2019). The phase I clinical trial provided interesting results. Firstly, at 24 and 36 months after administration of PVS-RIPO, the survival rate was 21%, which is significantly higher than in the historical control group. Secondlyp, the study confirmed the lack of neurovirulent potential, including paralysis associated with poliomyelitis (Desjardins, 2018; Iorgulescu, 2018).

Toca 511 is a modified retrovirus that expresses cytosine deaminase (McGranahan, 2019). It is a nonlytic virus, and its mechanism of action involves the conversion reaction of 5-fluorocytosine using cytosine deaminase to 5-fluorouracil, an antitumour drug of the antimetabolite group (McGranahan, 2019; Stepanenko, 2018). This is a replication-capable vector based on a mouse leukaemia virus-expressing enzyme of yeast origin (Stepanenko, 2018). A study by Mitschell et al. (Mitchell, 2017) carried out on a mouse model of glioma demonstrated that when Toca 511 was administered together with the prodrug 5-flourocytosine in animals, there was a change in the qualitative composition of the cells of the immune system present in the tumour environment. A loss of immunosuppressive cells and an expansion of T lymphocytes deep into the tumour were observed. Application of Toca 511 and 5-fluorocytosine resulted not only in tumour cell death, but also in the infiltration of immune cells deep into the tumour, altering the tumour microenvironment, which promotes the generation of anti-tumour immunity (Mitchell, 2017). Similarly, Yagiz et al. (Yagiz, 2016) tested in mouse and rat models the combination of Toca 511, 5-fluorouracil and another alkylating anticancer drug, lomustine, also obtaining satisfactory results in terms of survival (Yagiz, 2016).

Toca 511 has also been evaluated in clinical trials. In a phase I clinical trial, Toca 511 was administered into the cavity after tumour resection, followed by oral administration of 5-fluo-

rocytosine. 23 of 56 patients responded to the recommended dose. In this subgroup, the median overall survival was 14.4 months. Moreover, one-year survival rates were 65.2%, while the two-year survival rate was 34.8 %. Furthermore, as of 25 August 2017, five patients showed a complete response and were alive 33.9-52.2 months after administration (Cloughesy, 2018; Stepanenko, 2018). To gather more data, a clinical trial was conducted to compare the efficacy of treatment using Toca 511 with oral 5-fluorocytosine with standard treatments in patients who have glioblastoma multiforme or anaplastic astrocytoma and have undergone tumour resection for a first or second relapse. However, the results of these studies did not yield significant differences in survival between the patient groups studied (Cloughesy, 2020). Nevertheless, the results of the phase I clinical trial offer much hope for the development of effective treatments for glioblastoma multiforme.

Certainly, the results of studies on the use of virotherapy in the treatment of glioblastoma multiforme seem promising and may prove to be the future of therapy for this type of cancer. However, there is undoubtedly a need for more research in this area, both preclinical and clinical. Nevertheless, the innovation of the described therapies is also evidenced by the fact that the FDA has granted "Fast Track" status to both Toca 511 and PVS- RIPO and DNX-2401 (Martikainen, 2019).

CHECKPOINTS INHIBITORS

Another approach uses immune checkpoints inhibitors such as programmed death receptor 1 (PD-1), programmed death-ligand 1 (PD-L1), or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (McGranahan; 2019). The therapy using monoclonal antibodies directed at blocking immune checkpoints has proven effective in several types of cancer (Darvin, 2018). This raises hope also for use in the treatment of glioblastoma multiforme. Immune checkpoints cause inhibition of the antitumour immune response. Their blockade should therefore lead to an enhanced immune response and elimination of tumour cells (Darvin, 2018). PD-1 is a molecule that is expressed on immune system components such as T cells, B cells, bone marrow and NK cells. It has its biological effects by interacting with specific PD-L1 ligands in peripheral tissues. High levels of PD-1 and its ligands are associated with, among other things, impaired cytotoxic function of T cells and their secretion of cytokines (Maxwell, 2017). Several studies have demonstrated that PD-L1 is highly expressed in glioblastoma, which may prove to be a good target for therapy, in addition, studies show that increased PD-L1 levels correlate with increased susceptibility to treatment using immune checkpoint inhibition (McGranahan, 2019; Wang, 2019). One molecule registered to block PD-1 is nivolumab, a fully humanised monoclonal antibody. Currently, this drug is used in the treatment of melanoma, non-small cell lung cancer, and kidney cancer, among others (Koseła-Paterczyk, 2016). Other monoclonal antibodies directed against PD-1 or PD-L1 include pembrolizumab, durvalumab and atezolizumab (Yang, 2021). Immune check-point inhibitors have achieved good results in preclinical studies in GBM, but their potential in therapy is limited because of the risk of central nervous system (CNS) toxicity (McGranahan, 2019).

Several clinical trials have investigated the efficacy of immune checkpoint inhibitors in the treatment of glioblastoma multiforme. One of these studies examined the efficacy of nivolumab or pembrolizumab in patients with recurrent highgrade glioma. In this study, some patients were also taking bevacizumab, an anti-angiogenic agent used in cancer therapy. Unfortunately, the results showed no survival benefit (Kurz, 2018). Subsequent studies have also failed to provide groundbreaking evidence of the efficacy of immune checkpoint inhibitors in the treatment of GBM. The CheckMate 143 trial compared the efficacy of nivolumab and bevacizumab in patients with first relapse who had previously received temozolomide and radiotherapy. Unfortunately, this study showed no improvement in overall survival in the group of patients treated with nivolumab relative to the group receiving bevacizumab (Reardon, 2017). In contrast, Lukas et al. (Lucas, 2018) studied the efficacy of atezolimumab in patients with glioblastoma multiforme at first or second relapse. The drug was well tolerated, but the 12-months overall survival time was comparable to that with chemotherapy or bevacizumab.

Although the development of immune checkpoint inhibitors has revolutionised the treatment of some cancers, the future of their use in the treatment of glioblastoma multi-forme is unclear. The results of clinical trials of the use of single immune checkpoint inhibitors have not provided breakthrough data. This may be due in part

to the immu-nosuppressive environment of this tumour, including but not limited to a reduced number of lymphocytes infiltrating the tumour. Therapy failures may also be related to PD-L1 expression in tumour cells, the number of mutations, or microsatellite instability (Yang, 2021). Consequently, combination therapies – which include the use of more inhibitors of different checkpoints – are being investigated. Combination therapies using immune checkpoint inhibitors are discussed later in this article.

COMBINATION THERAPIES

Currently, classical therapies for glioblastoma multiforme focus on surgical treatment or the use of chemotherapy or radiotherapy. All of these methods place a heavy burden on the patient, which is why new therapeutic options are increasingly being researched (Huang, 2021). An example of such a method is immunotherapy which includes a number of different technologies, i.e. the use of monoclonal antibodies, CAR-T therapy or the use of checkpoint inhibitors (Chan, 2021; Huang, 2021). Using only one of these methods is called monotherapy. Often this choice of treatment is insufficient, so current clinical trials will largely focus on the possibility of combining different therapies. This significantly increases therapeutic options, leading to a synergistic effect of two or more therapeutic substances. Of greatest interest among researchers have been combinations of immunotherapy together with CAR-T therapy or multiple checkpoint inhibitors, but also immunotherapy with radiotherapy (Chan, 2021; Huang, 2021).

The combination of radiotherapy and immunotherapy offers promising opportunities. It is assumed that adequate doses of radiation are able to lead to tumour necrosis by triggering the abscopal effect which has been described in detail by Mole et al. (De Martino, 2021; Medikonda, 2021). In addition, the presentation of antigens and neo-antigenes is increased, resulting in immunomodulation. Ongoing clinical trials are currently focused on evaluating the safety of using a combination of hypofractionated stereotactic radiotherapy (HFSRT) with immunotherapy, mainly with anti-PD-1 monoclonal antibodies (Medikonda, 2021; Sahebjam, 2021). The clinical trial comprised 32 patients who were treated with bevacizumab plus pembrolizumab with concomitant HFSRT (Sahebjam, 2021). The results confirmed the safety of immunotherapy combined with radiotherapy. Significant PD-1 expression ≥ 10% was observed in only one of the patients studied, suggesting the high efficacy of this treatment method (Sahebjam, 2021). In addition, researchers are focusing on combining laser ablation with the simultaneous use of anti- PD-1 monoclonal antibodies in the treatment of GBM. It is suggested that with this method it is possible to eliminate the main problem of treating brain tumours, i.e. the blood-brain barrier (Medikonda, 2021). As a result of the loss of this barrier, there may be an influx of immune cells into the tumour and there is also an increase in the availability of tumour antigens, but the safety of this method is still under investigation (Medikonda, 2021).

An interesting idea with promising preliminary results from clinical and preclinical studies is the possibility of using a combination of multiple checkpoint inhibitors, i.e. PD-1, CTLA-4, indoleamine 2,3-dioxygenase 1 (IDO1) and a combination of lympho-cyte-activation gene 3 (LAG-3) and T-cell immunoglobulin and mucin-domain con-taining-3 (TIM-3) with anti-PD-1. The concept of combination therapy has currently been approved by the FDA for the treatment of melanoma thanks to the use of PD-1 and CTLA-4 inhibitors. Researchers suppose that such a combination is also worth considering for the treatment of glioblastoma multiforme. Thus, preclinical phase studies are currently being conducted on the utility of these inhibitors against GBM (Chan, 2021). One of the characteristic checkpoint molecules for glioblastoma multiforme is the IDO1 molecule. Its function described to date is to convert the amino acid tryptophan to kynurenine which has an immunosuppressive role and additionally increases salience to metastasis (Chan, 2021; Zhai, 2021). Increased expression of the IDO1 molecule is also correlated with higher mortality by increasing the influx of immunosuppressive Treg cells. Therefore, a clinical trial was conducted using triple checkpoint blockade, i.e. PD-1, CTLA-4 and IDO1. The results showed that under the application of this antibody combination, there was a significant reduction of Treg cells within the tumour (Chan, 2021). Similar effects in preclinical studies were obtained with the combination of anti-PD-1 inhibitors with LAG-3 and anti-PD-1 with TIM-3 (Chan, 2021).

In the treatment strategy for glioblastoma multiforme, combinations of modern cancer therapy

regimens are also being considered. An example of such a combination is the use of the previously described CAR-T system with checkpoint inhibitors (Chan, 2021; Maggs, 2021). The use of these inhibitors as a monotherapy is often limited due to the fact that a T-cell response is required and also through the presentation of neoantigens by class I tissue compatibility system antigens (Maggs, 2021). In ongoing preclinical studies on glioblastoma multiforme models, anti-PD-1 and anti-CTLA-4 checkpoint inhibitors were used in combination with CAR-T cells. In the first case, CAR-T cells containing the extracellular domain of anti- IL13Rα2 were used in combination with ipilimumab and nivolumab to assess the safety and the lack of toxicity on body cells excluding tumour cells. The postulated synergistic effect of this therapy is to help immune cells fight the tumour and also lead to the inhibition of tumour cell growth and metastasis (Maggs, 2021). The combination of anti-EGFRvIII CAR-T cells with an anti-PD-1 monoclonal antibody is also one of the similar studies currently underway (Maggs, 2021).

The possibility of combining several therapies offers great opportunities for the treat-ment of glioblastoma multiforme. The main problem, however, continues to be the blood-brain barrier, through which only selected molecules can pass. Researchers are therefore attempting to combine the therapeutic methods available to date for different types of cancer in order to effectively bypass this obstacle. In addition to the described combinations of different methods, clinical or preclinical studies are conducted combining immunotherapy with protein vaccines, chemo-therapy or epigenetic drugs (Chan, 2021; Maggs, 2021) as well as with nanotechnology in its broad sense, thanks to which it is possible to deliver a therapeutic substance to a precisely targeted place within a given tumour (Maggs, 2021).

SHORT CONCLUSION

Immunotherapy has changed the lives of many cancer patients. Research into its use in glioblastoma multiforme is extremely important, as standard treatments do not extend the life span of patients very much. Due to the complex biology of glioblastoma multiforme, it is difficult to find an effective therapy, as this is related not only to the high heterogeneity within the tumour, but also between individual patients. The low mutational burden of the tumour is also unfavourable. Furthermore, the immunosuppressive microenvironment of GBM is also responsible for immunotherapy failures. However, data coming from an increasing number of clinical trials raise hope. The use of different types of immunotherapy, such as cancer vaccines, CAR-T therapy, virotherapy or immune checkpoint inhibitors has proven effective in some patients. Even a slight prolongation of survival among patients is a significant achievement. Moreover, immunotherapy seems to be much more effective when not one, but more strategies are used. In addition, the results of trials of combination therapies using both immunotherapy and other treatments seem very promising. Undoubtedly, more research is needed, both on the biology of glioblastoma multiforme itself, which will allow the development of patient-specific personalised therapies, and on the development of new combination therapy regimens.

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Prevention of cervical cancer in Poland before and during the COVID-19 pandemic

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ABSTRACT

Cytodiagnostic prophylaxis programs conducted in recent years have contributed to a significant decrease in the incidence of cervical cancer. Obtaining results in the form of effective screening required years of work, constant financial outlays, quality control of the actions taken, as well as systematicity and cooperation of multidisciplinary teams of doctors, midwives and many institutions. While the time before the outbreak of the COVID-19 pandemic brought positive effects of such actions, the pandemic caused a decrease in the number of tests performed and, consequently, an increase in the incidence of cervical cancer. The aim of this study is to present the scope of activities included in cervical cancer prevention programs in Poland before and during the COVID-19 pandemic and to show the relationship between the effectiveness of preventive activities and the incidence of cervical cancer among women.

Keywords: prevention, cervical cancer, COVID-19

INTRODUCTION

Recent years in medicine have been a time when the huge role of preventive measures has been emphasised. Undoubtedly, the development of vaccines revolutionized the world and became a breakthrough in the prevention and treatment of many diseases. Cervical cancer fits perfectly into all concepts of prevention (Curyło-Sikora, 2016). A distinction is made between primary and secondary prophylaxis, and preventive examinations have an impact on reducing mortality.

The disease entity of cervical cancer is the first type of human cancer for which triggers and predisposing factors have been defined. Numerous studies have unequivocally demonstrated a link with infections, especially oncogenic strains of HPV (*Human Papilloma Virus*). It is believed that more than 99% of cervical cancers are linked to human papillomavirus infections, although 80% of all HPV infections are self-cured. This is because only about 20% of viruses have the ability to complete their replication cycle (Bręborowicz, 2016).

Numerous studies indicate that cervical cancer develops in 3-5% of infected women. This process, untreated, progresses within 8-12 years to the so-called invasive stage, when cancer cells begin to penetrate the basement membrane of the epithelium and invade the cervical wall. They have the ability to form metastatic foci via lymphatic and blood vessels.

It is well known that the sexually transmitted HPV virus often causes chronic infection with high-goncogenic types of this virus. It is both the main and the most important of all risk factors for cervical cancer. It often acts as a promoter. In contrast, other viruses, such as herpes virus and bacteria, only play a role in the process of carcinogenesis. In addition, factors such as nicotinism, early initiation of sexual intercourse or a large number of sexual partners, low socioeconomic status, neglect of personal hygiene, longterm use of oral hormonal contraception, or hormone replacement therapy stand out among the factors that increase the likelihood of persistent infection (Faridi, 2016).

As a response to the problem of high incidence and mortality of cervical cancer world-wide, it became possible to introduce the HPV vaccine (primary prevention) into widespread use, aimed at reducing the aforementioned rates (Wójtowicz, 2016), and to develop cytological screening and HPV testing (secondary prevention). Very often cytological screening is performed in combination with HPV testing (Dębski, 2009).

The overriding aim of prophylactic tests, which cover the entire healthy population, has become the earliest possible detection of cancer and the rapid initiation of appropriate, effective treatment. Cure, and thus lowering the mortality rate, has also become the top priority for prevention (Stępień, 2011).

PROPHYLAXIS OF CERVICAL CANCER IN THE PERIOD BEFORE THE OUTBREAK OF THE COVID-19 PANDEMIC

The cervical cancer prevention programme has been operating in Poland since 2006. It is aimed at women between 25 and 59 years of age who are insured in the National Health Fund (NFZ), including women who have not undergone such examinations within the last three years. The exceptions are women who were referred for an addi-tional examination because of changes detected in the primary examination. It should be remembered that the available statistics do not include the number of tests performed outside the programme in private practices and clinics that do not cooperate with the NFZ. Women who undergo tests in such places are therefore not registered in the general database, which means that they are not fully monitored.

Cytological examinations are carried out every three years and are supposed to reduce the number of infections by up to 90%. Cytological examinations are recommended to women who are at least 21 years old, but not later than in the third year from the beginning of sexual inter-course by a woman. However, due to the high percentage of false-positive results in younger women, i.e. 21-25 years of age, it is not recom-mended to carry out tests and implement deeper diagnostics, as this age group has a low risk of developing cancer. The upper limit in Poland for carrying out prophylactic cytological exami-nations is when a woman reaches the age of 60 (Stępień, 2011).

Data from 2010, when the programme was in its early stages, shows that the enrolment of women for screening was very low. The rate depended on the place of residence. At that time the per-centage of inhabitants of particular provinces who responded to personal invitations to cervical cancer screening was appallingly low and amounted to approximately 9%. Whereas, within the framework of the whole population scre-ening for early detection of cervical cancer, the number of all examinations performed was only 25%.

For the first time, in addition to the undoubted need and willingness to carry out mass and active screening programmes, financial resources were allocated for this purpose, making it possible, it would seem, to carry them out effec-tively. Numerous diagnostic bases have been created, operating on the basis of health pro-grammes, particularly active in the years 2014-2019. Citing the results of other countries, applying controlled cytodiagnosis covering at least 2/3 of the population of women examined, in which an 80% decrease in the incidence of cervical cancer has been achieved, as well as, significantly, a 70% decrease in mortality (Jemal, 2011) – cytological screening has also become in Poland a basic and necessary element of prevention and early detection of cervical cancer (Spaczyński, 2009).

There has been considerable interest in proph-lactic activities in Poland, though the reporting rates for prophylactic examinations and the number of examinations performed have not been satisfactory and have varied depending on the region of the country. In different parts of Poland, at different times, in addition to the implementation of the programme, numerous promotional activities and educational meetings were held in hospitals, outpatient clinics and gynaecological surgeries, involving medical professionals and local authorities.

According to the data obtained from the imple-mentation of the population-based programme of cervical cancer prevention and early detection within the framework of the National Programme for Combating Cancer Diseases for the years 2007-2010, three provinces were characterised by the highest enrolment of women covered by the exa-minations in 2007: namely Warmińsko-Mazurskie (41.58%) in first place, followed by Opolskie (32.15%) and Pomorskie (29.31%). The lowest notifiability was in Lubuskie (15.88%), Podkarpackie (12.04%) and finally Wielkopolskie (10.43%). The situation was similar in 2008, when Warmińsko-Mazurskie (33.59%) and Opolskie (30.97%) provinces also led in the reporting of the female population. The lowest percentage of notifications also occurred in the Wielkopolskie (18.73%), Łódzkie (19.04%) and Mazowieckie (18.12%) provinces. In subsequent years, the Warmińsko-Mazurskie (2009-33.88%, 2010-26.49%) and Opolskie (2009-32.38%, 2010-22.73%) provi-nces were also leaders in the implementation of the population-based prophylaxis of cervical cancer, although to a lesser extent. The worst results were obtained in Wielkopolskie province (2009-19.76%, 2010-12.56%). Thus, the highest percentage of women's participation in the programme in the years 2007-2010 was in the

Warmińsko-Mazurskie, Zachodniopomorskie, Opolskie provinces, with the greatest differences in 2007. The lowest percentage of the examined population could be observed in the Wielkopolskie, Mazowieckie and Podkarpackie provinces (Spaczyński M., 2010).

Effective cervical cancer prevention strategies were based on international experience and recommendations in the three most important areas. These concerned cytological examinations, vaccinations and educating the public on the dangers of cancer and HPV and the possibilities of effective prevention. In connection with primary prevention, which concerned vaccination, and secondary prevention concerning cytology, education played a special role. The assumption was to address it to as many addressees as possible and to conduct it as to build public awareness and to mobilise individuals to take part in examinations. It was considered to be the most important factor influencing the effectiveness of prevention.

Actions taken in Poland as part of HPV prevention have been implemented by many institutions, which had not yet been fully compatible. That is why implementing a coherent, common strategy was difficult. In Poland, among the three pillars of cervical cancer prevention, most attention has so far been paid to cytological examinations, while issues of vaccination have depended on the region of the country (implementation of local government programs). HPV vaccination, which has been on the list of recommended vaccinations since 2013, but unfortunately not publicly funded. Only some local government prevention programmes have carried out free HPV vaccination. An interesting form of program in the prevention of HPV papillomavirus infection, referring to international recommendations concerning prevention based on three pillars, was implemented by the Warmian-Masurian Voivodship, which for a number of years was leading among other voivodships in the prevention of cervical cancer. It was introduced under the name "Health Policy Programme of the Warmińsko-Mazurskie Voivodeship for 2017-2019 in the prevention of human papillomavirus (HPV) infections – in particular, these were educational activities and vaccination of girls aged 11-13 years", to complement the national intervention. In the Warmińsko-Mazurskie Voivodship the incidence of cervical cancer in comparison to other voivodships remains at a moderate level.

Worldwide, 530,000 new cases were reported in 2012, while over 270,000 women died of the disease during the year. The high proportion of patients with cervical cancer in advanced stages of the disease (stage II, III, IV), placed Poland on one of the first places in Europe in this respect. This situation was associated with the generally poor state of health and life of Polish society, as well as with ineffective prophylactic measures of the health service at that time in comparison with other European countries. As a result, only about 12% of women of reproductive age were covered by cytooncological prophylaxis. The lack of an appropriate lobby which could exert political and social pressure to change the global health care strategy and the low socio-economic status of the patient population were also reasons for this situation.

According to data from the National Cancer Registry, in recent years in Poland, before the outbreak of the pandemic, there was a noticeable increase in the number of cases in women aged 35-44, as well as one of the lowest 5-year survival rates in Europe as a measure of the curability of this cancer. It was quite high at 48.3% against a European average of 62.1%. The cure rate, as previously mentioned, depends primarily on the stage of the cervical cancer at the time of diagnosis and the type of microscopic structure, the degree of maturity of the tumour and the depth of involvement of the uterine tissues, as well as the presence of lymph node metastases. According to the same registry, in 2013, 2909 women developed cervical malignancies, and the percentage of incidence according to the statistics has decreased by about 30% over the last three decades, which did not change the fact that the rates still remained high and still represented a serious epidemiological problem.

Subsequent years still brought new cases (about 3450 per year), despite the ongoing and widely available cytological prophylactic examinations and the increasing access to diagnostic tests. The problem was to a large extent caused by low social awareness, concerning the lack of the habit of prophylactic examinations and low knowledge about the possibilities of full cure of the disease in the case of early detection of cervical cancer (Stępień, 2011). That is why prophylactic programs and social campaigns aimed at raising public awareness and encouraging active prophylaxis are very important.

PREVENTION OF CERVICAL CANCER IN THE ERA OF COVID-19 PANDEMIC

The standardised death rate from cervical cancer has improved steadily in recent years. In 20 years, it has decreased from 8.74 (1990) to 5.57 (2018), highlighting the effects of the last five years, when a decrease of 13.8% was recorded (Wojciechowska, 2021). Due to the possibility of using effective intervention methods to reduce mortality from cancer, including cervical cancer – the National Program for the Control of Cancer was announced in 2005. However, the main objectives, relating to increasing screening attendance and thus achieving average European treatment success rates, were not achieved (Wojtyniak, 2018). Increasing screening attendance became the main objec-tive of the National Cancer Strategy, adopted in February 2020, with the aim of achieving rates of 60% in 2024. On the other hand, from March 2020, due to the epidemic situation, restrictions and limitations in social life and access to medical services began to be introduced. The development of the pandemic exposed the extremely difficult health situation of the population at that time in terms of the implementation of preventive programmes and chronically ill patients – including oncology patients, which are constantly increasing (Ramirez, 2020). Patients' fears of coronavirus infection and the suspension of preventive, diagnostic and treatment activities, associated with limited access to medical services, have contributed to this. For the past two years, the public attention of the entire world, has been drawn to the ever-increasing number of new cases of coronavirus infection, and the appeals of oncology specialists to ensure continuity of care for oncology patients have fallen by the wayside. Today, many researchers point out that the victims of the pandemic are not only the infected, but also people with other conditions and those who have been prevented or delayed from having preventive tests by the pandemic.

According to the data, in 2020 there were 3862 new cases of cervical cancer in Poland and 2137 deaths (11/100 000). For comparison, this disease entity in Poland ranks 6th among malignant neoplasms, while in Europe it ranks 9th in terms of incidence and 10th in terms of mortality (6.7/100 000) (Globocan, 2020).

Given that vaccination and screening among vaccinated and unvaccinated women is considered the best and most effective strategy for cervical cancer prevention, the results show that the timing of the pandemic may have adversely affected the statistics. While a few years before the outbreak of the pandemic may have seen slow but positive changes in the improvement of statistics relating to incidence and screening rates, the timing of the pandemic had an adverse effect. Restrictions and restrictions have made it more difficult to access basic medical services, including the possibility of carrying out preventive tests. It turns out that it is precisely prophylaxis that is the most affected area of Polish oncology.

The pandemic has affected oncological care in a heterogeneous way. The most significant changes in comparison to previous years in terms of detection, diagnosis, medical services occurred at the level of primary care and AOS in the period from March to May 2020. During this period, intensive activities were carried out throughout Poland to prevent the rapid spread of the epidemic. In addition, during this period there was general confusion about the information on the spread of the virus and public fear of SARS-CoV-2 infection.

According to the 2021 Report on "The Impact Of the COVID-19 Pandemic On the Cancer Care System", the number of new cancer diagnoses during this time dropped by approximately 20% in 2020 compared to 2019. Similar values were reported in other European countries. This report was created based on the analysis of data obtained from three provinces: Warmińsko-Mazurskie, Mazowieckie and Śląskie for the periods of March, April, May 2019 and identical months in 2020. These regions differed significantly in the number of confirmed infections and cancer incidence. An analysis was made in terms of continuity of services in oncology, where, among other things, one of the elements examined, was preventive examinations for cervical cancer. According to the information obtained, the epidemiological situation caused the number of cytological examinations to decrease by 60% in all three voivodeships in the month of March 2020. In the following two months, this percentage increased respectively: Warmińsko-Mazurskie 85%, Mazowieckie 87% and to 90% in Śląskie. Comparing month to month in the period January-February 2020, at the time of the growing number of infections, the individual provinces recorded the following percentage decreases in the number of tests performed:

Silesia -16.3%, Mazovia -12.6%, Warmia and Masuria -17.1%. In the period April-May 2020: Silesia -90.2%, Mazovia -87.0% and Warmia and Masuria -84.8%. This indicates a significant decrease in the number of completed surveys under the programme for all the analysed voivodeships.

Table 1. Summary of cytological tests performed in 2019-2020 according to the Report "Oncology in the time of COVID-19"

PAP SMEARS 2019 vs.2020					
	January	February	March	April	May
Śląsk	-22,7%	-4,7%	-61,0%	-95,8%	-84,8%
Mazowsze	-16,3%	-9,1%	-62,4%	-94,8%	-79,3%
Warmia and Mazury	-22,3%	-11,8%	-61,5%	-91,1%	-78,0%
	January-February			April-May	
Śląsk	-13,6%			-90,2%	
Mazowsze	-12,6%			-87,0%	
Warmia and Mazury	-17,1%			-84,8%	

It is worth mentioning that the analysis did not include examinations performed in private offices. Nevertheless, the statistics for this period are unsatisfactory and we still have to wait to learn about the longterm effects.

Preventive programmes against cervical cancer and other cancerous diseases are part of the WHO strategy "Health for All In the 21st Century", which concerns the reduction of non-communicable diseases. This strategy assumes maximum reduction of morbidity, disability and premature mortality due to chronic diseases, among others due to cervical cancer, the mortality rate of which in Poland is still at a high level and in this respect is among the top ten countries. According to the recommendations of the Expert Panel of the Polish Gynecological Society (PTG), the cervical cancer prevention programme should be implemented based on the principle: mother – cytological screening, daughter – vaccination against HPV. Therefore, cervical cancer prevention should be interdisciplinary, involving education and social activation as well as vaccination, screening, treatment and palliative care. Successive waves of the pandemic have evidently stopped or hindered education and research on cervical cancer prevention. This has placed new demands on medical professionals as well as on the country's health care system as a whole.

CONCLUSIONS

The onset of the pandemic is closely associated with a decrease in the number of cytological examinations performed in Poland. In the case of the individual voivodships of the country under discussion, there was a decrease in preventive measures at the same level. Therefore, this fact can certainly be associated with the consequences of the COVID-19 pandemic and the restrictions in place at the time.

Preventive screenings are fundamental in the fight against cervical cancer. The reporting and performance of cytological examinations is closely linked to women's awareness of the benefits of regular examinations. The COVID-19 pandemic, the associated restrictions and the public's fear of contagion have had an impact on the reporting and performance of cytological examinations, which allows us to conclude that the number of preventive examinations for cervical cancer decreased during the pandemic period, which may affect the fate and prognosis of women when cancerous lesions are detected at an advanced stage of the disease.

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The role of epithelial-mesenchymal transition in the progression of pancreatic and colorectal malignancies

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Pracę wykonano w ramach projektu PW/Z/1/1/20(1) realizowanego w latach 2020-2021 finansowanego ze środków subwencji przeznaczonej na naukę uzyskanych przez War-szawski Uniwersytet Medyczny.

ABSTRACT

Epithelial-mesenchymal transition (EMT) is a morphologic cellular programme defined as the phenotypic transition from an epithelial to a mesenchymal state. Pathologically hyperactivated EMT is widely described in tumour development and progression. Proteins that orchestrate EMT have been correlated with increasing histologic grade and poor prognosis for several types of carcinomas. Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive solid malignancies, characterized by its insensitivity to the current therapy. Colorectal cancer (CRC) is the second most diagnosed malignancy among females and third among males worldwide. To provide the most effective diagnostic and therapeutic schemes for aforementioned carcinomas, new markers must be discovered and implemented. Various studies suggest that EMT is an indispensable process for PDAC and CDC growth and dissemination. Discovering new molecular models associated with the complex process of carcinogenesis lets us be a one step closer to more personalized diagnostic and treatment schemes for oncological patients. As an example, broaden research on the mechanism of pharmacological miRNA targeting might enable the future implementation of miRNA-based therapeutics into the CRC treatment schemes. On the other hand, proteins or genes related to EMT might become tempting targets for defeating chemo- or radioresistance in patients with PDAC. In addition, EMT inhibition or reversion might overcome acquired resistance to the implemented treatment caused by drug induced-EMT, both in PDAC and CRC patients. This study aims to sum up recent knowledge on the subject concerning epithelial-mesenchymal transition in the pancreatic and colorectal malignancies progression.

INTRODUCTION

Epithelial-mesenchymal transition (EMT) is a morphologic cellular programme defined as the phenotypic transition from an epithelial to a mesenchymal state. Pathologically hyperactivated EMT is widely described in tumour development and progression (Cho, 2019). During this reversible process, epithelial cells lose their cobblestone appearance in monolayer to acquire spindle-shaped mesenchymal morphology (Georgakopoulos-Soares, 2020). The transition is associated with repressing epithelial markers such as E-cadherin and overexpressing markers correlated with mesenchymal state, especially N-cadherin and vimentin. Cancer cells undergoing EMT are suitable for migration, invasion, and proliferation, thereby facilitating tumour progression (Dongre, 2019, Lamouille, 2014). EMT is driven by numerous transcription factors, including SNAIL, TWIST and zinc-finger E-box-binding (ZEB) that repress epithelial marker genes and activate genes associated with the mesenchymal phenotype (Lamouille, 2014). Proteins that orchestrate EMT have been correlated with increasing histologic grade and poor prognosis for several types of carcinomas. Regarding metastasis, numerous studies have shown that most circulating tumour cells express both epithelial and mesenchymal markers, emphasizing the crucial role of EMT during carcinoma dissemination (Ribatti, 2020, Aiello, 2019).

SEARCH STRATEGY AND SELECTION CRITERIA

The aim of the study was to collect up-to-date knowledge on the subject concerning epithelial-mesenchymal transition in the pancreatic and colorectal malignancies progression. To carry on the study, we used the following databases: PubMed, Google Scholar, Web of Science and Medline. The main search concept was to combine keyword "EMT" or "epithelial-mesenchymal transition" with related terms, such as "colorectal cancer", "CRC", "pancreatic cancer" or "PDAC". Particular attention has been concerned to English-language articles from the recent years, encompassing original papers, reviews, and case reports.

EMT IN THE PANCREATIC DUCTAL ADENOCARCINOMA

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive solid malignancies, characterised by its insensitivity to the current therapy (Singhi, 2019). The poor outcomes are mainly associated with the late presentation of the disease. Therefore, the detection of premalignant conditions is essential for early initiated treatment (Mcguigan, 2018). Various studies suggest that EMT is an indispensable process for pancreatic cancer growth and dissemination. EMT might occur even in the premalignant lesions, thus EMT markers might become crucial prognostic indicators (Wang, 2017). In the conducted studies several pancreatic cancer cell lines and surgically resected PDAC samples presented strong EMT characteristics (Cates, 2009, Kyuno, 2014). Immunohistochemical research performed on PDAC specimens indicated that median survival for patients with epithelial tumours was 40.2 months in comparison to 13.7 months for patients with mesenchymal phenotype. What is more, EMT markers status was associated with portal vein invasion and lymph node metastasis (Yamada, 2013).

Malignant tumours, including PDAC, consist of biologically heterogeneous cellular population. A small number of cells possess stem-cell-like characteristics, encompassing the ability to self-renew, which might lead to the tumour recurrence and drug-resistance (Ishiwata, 2018). Recently, the relationship between EMT phenotypes and cancer stem cells (CSCs) has been studied in samples derived from pancreatic cancer. It was proved that cells with EMT phenotype possess molecular characteristics of CSCs, whereas CSCs express an EMT phenotype (Zhou, 2017). Numerous researches confirmed the link between the acquisition of an EMT–like phenotype in cancer cells, CSCs and both chemo- and radioresistance (Niess, 2015; Yin, 2011). In the study conducted by Yin et al. (2011) pancreatic CSCs demonstrated an EMT phenotype, increased motility, and resistance to the gemcitabine-based treatment (Yin, 2011).

The substantial correlation between the epithelial-mesenchymal transition and systemic aggressiveness and drug-resistance has created novel perspectives for therapeutic agents for PDAC (Beuran, 2015). In the study by Polireddy et al. (2016) the induction of EMT resulted in increased drug resistance, metastasis, and elevated number of CSCs. Stem cell markers cannot be used as a markers source due to their limited expression in the very small proportion of cells. Looking for EMT inhibitor brings the possibility for discovering CSC inhibitor and in result overpassing cancer dissemination and drugresistance (Polireddy, 2016). As an example, in the study by Boreddy et al. (2013) deguelin targeted EMT by significant downregulation of the mesenchymal proteins and upregulation of epithelial markers both *in vivo* and *in vitro*. In result, deguelin inhibited metastasis of PDAC along with primary tumour growth (Boreddy, 2013). El Amrani et al. (2019) proved that gemcitabine treatment might induce EMT-like changes that maintain invasion and chemoresistance in PDAC cells (El Amrani, 2019). This discovery was consisted with previous research distinguishing chemosensitive cells with epithelial-like phenotype from more chemoresistant cells with mesenchymal-like phenotype (Kim, 2014).

Another approach concerns targeting tight junctions associated with EMT. In pancreatic cancer samples a few tight junction proteins are proved to be abnormally regulated. Claudin-1, protein kinase C and marvelD3, among others, are involved in EMT of pancreatic cancer cells thus they might become useful biomarkers during disease (Kyuno, 2014). MarvelD3 was proved to be transcriptionally downregulated in poorly differentiated pancreatic cancer cells and during Snail-induced EMT. Depletion of marvelD3 resulted in a decrease in transepithelial electrical resistance and in an increase of permeability (Kojima, 2011). For developing innovative diagnostic and therapeutic schemes via tight junction molecules it seems necessary to investigate the profile and the regulation of tight junctions in pancreatic cancer cells and compare different families, e.g., claudins and MARVEL (Kojima, 2011, Kyuno, 2014).

EMT IN THE COLORECTAL CANCER

Colorectal cancer (CRC) is the second most diagnosed malignancy among females and third among males worldwide (Ghoncheh, 2016). A significant number of patients with CRC undergoing operation unfortunately develop local recurrence or distant metastasis leading to shorter survival. Despite the development of

treatment regimens, there is no effective therapy for advanced CRC (Vu, 2017).

Clinical studies concerning CRC proved that diffuse positivity of the tumour cells for the EMT markers is correlated with unfavourable prognosis. In the study by Shioiri et al. (2006) Slug expression was significantly associated with Dukes stage and distant metastasis. Moreover, E-cadherin expression was significantly correlated with depth of tumour, lymph node metastasis, and Dukes stage (Shioiri, 2006). EMT was linked to the mobility and dissemination of CRC by conferring increased invasiveness and cells metastatic potential (Qi, 2014, Zhang, 2014). In the study by Deng et al. (2016) Twist (a transcription factor regulating EMT) overexpression triggered EMT and a CSC-like phenotype in human colorectal cancer cells and enhanced their migration and invasion. Additionally, Twist-overexpressing CRC cells presented stronger chemoresistance to the oxaliplatin than control samples (Deng, 2016).

A relatively new aspect described in terms of CRC concerns drug induced EMT. Oxaliplatin is suggested to induce the EMT by promoting the release of reactive oxygen species (ROS). Pretreatment with the ROS scavenger N-acetyl-L-cysteine inhibits oxaliplatin-induced EMT and metastasis (Jiao, 2016). On the other hand, another study proved that radiotherapy induces an alteration to a malignant phenotype consistent with EMT in colorectal cancer cells (Kawamoto, 2012). In the research conducted on SW480 CRC cells increased radiation was correlated with mesenchymal phenotype and enhanced migration and invasion abilities (Lin, 2017). However, the radiosensitivity of CRC might be enhanced by Vitamin D via EMT reversing process. Vitamin D inhibits EMT through upregulating cystatin D (the E-cadherin inductor) and plasminogen activator inhibitor-1 and in result intensifies the radiation therapeutic effect on CRC (Yu, 2021).

MicroRNAs (miRNAs) are small endogenous RNAs regulating posttranscriptional silencing of target genes. Therapeutics based on these molecules represent one of the significant areas of scientists' interest because their involvement in carcinogenesis (Fudalej, 2021). The number of miRNAs regulate the epithelial phenotype and EMT by inhibiting the expression of EMT regulators in the cancer cells. Recent studies have demonstrated that the members of the miR-29 and miR-200 families are involved in the CRC progression by regulating epithelial-mesenchymal transition (Chi, 2016). The study by Hur et al. (2013) revealed that restoration of miR-200c inhibits migration and invasion in various CRC cell lines through direct targeting ZEB1, the transcriptional repressor of E-cadherin. It emphasized a pivotal role of miR-200c in the metastatic behaviour of CRC cells and suggests that miR-200c might become a potential diagnostic biomarker and therapeutic target (Hur, 2013). In the *in vitro* studies miR-29c was downregulated in CRC and suppressed EMT. It presented its role in cell migration by negative regulation of the Wnt/β-catenin signalling pathway (Zhang, 2014). On the other hand, increased expression of miR-29a promoted CRC metastasis by E-cadherin inhibition, which highlighted the potential of the miR-29a inhibitor as a novel therapeutic against CRC metastasis (Tang, 2014). Broaden research on the mechanism of pharmacological miRNA targeting might enable the future implementation of miRNA-based therapeutics into the CRC treatment schemes. However, focusing on a single miRNA brings a limited clinical approach, due to the complexity of miRNAs connections involved in the process of carcinogenesis (Dragomir, 2018).

CONCLUSION

Undeniably, EMT plays meaningful role in the growth, proliferation, and dissemination of pancreatic and colorectal malignancies. Selected markers associated with this process might be used in the future to develop and establish personalized diagnosis, risk stratification platform, and treatment algorithm for oncological patients, to improve their survival.

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Low-carbohydrate diet in glioblastoma treatment – mechanism of action and safety

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ABSTRACT

Gliomas are primary neuroepithelial neoplasms, i.e. originating from the central nervous system. They account for about 30-40% of brain tumors. 40 to 90% of gliomas are malignant neoplasms. Conventional therapeutic approaches often fail to provide cure or long-term remission. An additional factor supporting the treatment may be a diet based on changes in the metabolic environment of the body.

Healthy cells have the ability to change from glucose to ketone bodies as their main source of energy. It is an evolutionary adaptation to food scarcity that allows survival during extreme changes in the environment. Unlike normal neuronal and glia cells, which easily use ketone bodies (β-oxybate) for energy, brain tumors are highly dependent on glycolysis. The condition for the flexible transition of cells from glucose sources to ketone bodies is a correct genome and mitochondria. The situation that limits this possibility are cancer mutations. An approach to treating brain tumors that uses the metabolic flexibility of healthy brain cells at the expense of cancer cells could become the future for extending the life of this group of patients. Studies in both animal and human models confirm the benefits of implementing a low-carbohydrate diet in the treatment of glioblastoma.

This chapter summarizes the latest reports in this area. A low-carbohydrate diet brings not only benefits such as reduction of tumor size and prolonged survival of cancer patients, but also many doubts about the safety of its long-term use due to malnutrition, deficiencies and changes in the intestinal microflora.

INTRODUCTION

2% of all cancers are primary brain tumors and 30-40% of them are gliomas. Most often, gliomas are found in adults between the ages of 40 and 65. Ratio male-female is 6:4 (Zülch, 1986). About 50% of gliomas in adults are glioblastomas (Schneider, 2010). Glioma arising from the glial group is in four grade and the IV grade of glioma is glioblastoma which is the most aggressive form. Glioblastoma (GBM) is account about for over 82% of all malignant gliomas (Varshneya, 2015). The etiology of GMB for-mation is unknown. It is suspected that both endogenous and exogenous factors may play a role. The incidence of the disease depends on age, sex, genetic factors, origin, exposure to infections, viruses, radiation, exposure to certain chemicals and diet (McLean, 2005; Idowu1, 2008).

The standard therapy in the treatment of glioblastoma is maximum excision followed by radiation and chemotherapy (temozolomide) (Martin, 2020). Medium survival after diagnosis, it is about 15 months. After treatment, it is approx. 5 years, and after treatment and relapse, it is about 25 months. Therefore, there is an ever increasing need to develop new treatments and prevention of glioblastoma that can accompany standard procedures (Klein, 2020). Recent research indicates alternative methods such as progesterone therapy or nutritional therapy that can inhibit angiogenesis in cancer glial cells while inhibiting the growth of a malignant tumor.

Glioblastoma cells, unlike normal cortical cells of the brain, are much more sensitive to changes in the level of glucose in the body. When there is a very low blood glucose level in the blood serum, e.g. due to starvation, healthy brain cells use ketone bodies as a source of energy, while this metabolic pathway is unavailable for glioblastoma cells (Klein, 2020). Impaired metabolic pathways of cancer cells are described in the 1950s by Otto Warburg. The differences in the use of glucose resources between cancer cells and healthy cells were referred to as the "Warburg Effect". It concerns genetic changes related to the use of glucose in the process of glycolysis in cancer cells. The metabolic differences may be related to the loss of p53, followed by an increase in the activity of serine-threonine kinase (Akt), which results in the use of excess ATP (Abdelwahab, 2012). Mutations in cancer cells can be used to combat them by altering the metabolic environment. Reduced glucose levels and increased ketone bodies prevent proliferation and inhibit the growth rate of GMB cells in animal and human models (Martuscello, 2016; Syfried, 2011). Significant

reduction in environmental glucose levels causes apoptosis of GMB cells, but not of normal brain cells. Additionally, high levels of ketone bodies inhibit the growth of GMB on human cell lines. It can therefore be assumed that both low glucose levels and high ketone bodies have a beneficial effect in the treatment of glioblastoma, while their synergistic effect is most beneficial (Syfried, 2011; Mukherjee, 2004). Caloric restrictions are an additional factor supporting the nutritional treatment of KD. The combination of nutritional ketosis and calorie reduction has a pro-apoptotic, anti-angiogenic, and anti-inflammatory effect, reducing the expression of the mTOR effector in mice with an experimental malignant glioma. This mechanism reduces tumor size and growth and increases rodent survival (Klein, 2020). Some studies suggest that nutritional ketosis may reduce tumor metastasis (Poff, 2015). It is also known that ketosis increases the sensitivity of cancer cells to chemotherapy and radiotherapy (Klement, 2018). It has been proven that during KD, low insulin and IGF-1 levels inhibit the PI3K/AKT signaling pathway, which promotes apoptosis, reduces proliferation and angiogenesis of cancer cells (Pondel, 2020). In contrast, high glucose levels cause tumor growth and stimulate angiogenesis, and prolong tumor life in mice (Seyfried, 2012; Mukherjee, 2004). Patients with GMB showing hyperglycemia have shortened survival times (Derr, 2009).

To induce a state of ketosis, it is important to supply carbohydrates in the diet. Among the diets, we distinguish high-carbohydrate diets, the energy of which comes from carbohydrates in more than 45%, from 26-44% of a medium-carbohydrate diet, less than 26% are low-carbohydrate diets and very low-carbohydrate diets contain less than 10% of carbohydrates from total energy (Oh, 2020). A low-carbohydrate diet is one that provides 20-60 grams of carbohydrates per day. Among low-carbohydrate diets, we can distinguish the Atkins diet and various ketogenic diets (KD) (Last, 2006). The Atkins diet was created in 1970 and is less restrictive with regard to the amount of calories, proteins or fats consumed, although about 65% of the energy requirement should be in the form of fat (Kossoff, 2008). Among KD, we can distinguish the classic 4: 1 form, the ketogenic MCT (Medium Chain Triglycerides) diet and the ketogenic diet with a low glycemic index (IG). KD 4: 1 should contain 90% energy as fat, for KD MCT is 73% (including 30-60% medium chain triglycerides) (McLean, 2004). A low GI KD diet should contain about 60% energy from fat, and carbohydrate sources should have a GI lower than 50 (Neal, 2017). Initially, to achieve the state of nutritional ketosis, i.e. the concentration of ketone bodies in the range of 1-7 mM in the blood serum, should be consume up to 20 grams of carbohydrates a day, then this amount can be increased to 50 grams (Pondel, 2020). This level of ketone bodies does not change the pH of the blood. Low blood glucose concentration causes the body to produce small amounts of insulin and increased amounts of glucagon, which in turn leads to the use of free fatty acids (FFA) released together with glycerol from adipose tissue by adrenaline as a result of lipase (Longo, 2019). The released fatty acids are converted by the mitochondria of hepatocytes in the process of β-oxidation to acetyl-CoA. With low blood glucose levels during the Krebs cycle, the accumulated acetyl-CoA is used to produce ketone bodies. The three main ketone bodies are acetoacetate (AcAc), b-hydroxybutyrate (BHB) and acetone. The first two are the most important to produce energy for the body. Due to its volatile nature, acetone is eliminated through the lungs and through the kidneys. AcAc and BHB in the mitochondria of tissues other than liver tissues are converted into ATP in the Krebs cycle (Glew, 2010).

The ketogenic diet is a very demanding diet in many ways. It must be strictly respected not only by the sick person, but also by their caregiver. It often promotes caloric reduction, which can lead, along with elimination, to nutritional deficiencies and malnutrition. It should be remembered that not all the products allowed in it are generally considered healthy. The most common sources of fat in KD are oils, fatty meats and fish, fatty dairy products, including cream and cheese, and eggs. Additionally, the diet should be enriched with low-starch vegetables. There are special nutritional preparations available on the market that ensure the optimal composition of the diet and better control over ketosis (Freeman, 2010). The use of a ketogenic diet in the treatment of cancer depends on the type of cancer, its subtype, location, and genetic factors (Pondel, 2020). The studies carried out on both the animal and human models seem to be contradictory.

SEARCH STARTEGY AND SELECTION CRITERIA

The review contains all studies from 2007 to 2021 listed in PubMed for the search terms: ketogenic diet, diet, low-carbohydrate diet, cancer, glioblastoma, glioma. In the studies, the parameters relevant to the review were first and foremost, survival, tumor size, side effects, and group size. 12 studies on an animal model and 9 studies on a human model were selected. 3 case studies were also taken into account.

ANIMAL MODEL RESEARCH

Studies in animal models often show positive results. However, their result depends on many factors, which makes it even more difficult to decide whether KD therapy is effective and should be recommended in the treatment of GBM.

Nutritional ketosis is often used as an adjunct to conventional treatment. Abdelwahab et al. studied mice with GL-261 malignant glioma. Both in the group of animals undergoing radiotherapy and chemotherapy (temozolamide), their lives were prolonged during the use of KD compared to the control group (Abdelwahab 2012; Abdelwahab, 2011). Also, studies from 2014 showed that only a combination of diet and chemotherapy is an effective method to extend the life of sick animals. Without treatment, this effect was not observed for the KD group (Rieger, 2014). Another study also found that KD combined with caloric restriction, chemotherapy or a hyperbaric chamber increased survival. This has not been observed in the no-treatment group on either a ketogenic or calorie restricted diet (Augur, 2018). Similar results were obtained in another study where mice with malignant glioma on a low-calorie ketogenic diet were given 6-diazo-5-oxo-L-norleucine. In this group, tumor growth was inhibited and survival increased. No such correlations were observed in the control group on a standard diet treated with 6-diazo-5-oxo-L-norleucine. In this study there was no caloric deficit control group without Kd and treated with 6-diazo-5-oxo-L-norleucine, therefore we cannot exclude that caloric restriction and treatment are the most important factor (Mukherjee, 2019).

There are reports in animal studies relating to the successful use of KD in the treatment of GBM without chemotherapy or radiotherapy. This is confirmed by a study on mice that used the 8: 1 ketogenic diet. In this diet, the glucose level did not change, but the level of ketone bodies increased significantly, which resulted in a decrease of tumor progression, an increase of survival compared to the control group (Stafford, 2010; Scheck, 2012). The positive test results were explained by decrease ekspression of coding genes IGF-1, Ras GTPase activating protein (RasGAP) i mitogenactivated protein kinase 8 (MAPK8) in mause tumor. This resulted in changes in the signaling pathways of factors responsible for the growth of glioblastoma: IGF-1, platelet-derived growth factor (PDGF) and epidermal growth factor receptor (EGFR) (Scheck 2012). Similar results were also obtained by Lussier et al. using dietary intervention in mice with GL-261 malignant glioma. The anti-tumor effect was achieved in comparison with the control group (Lussier 2016). However, in another study, KD reduced microcir-culation of a malignant tumor in mice (Woolf, 2015). Also, positive results KD shows research with three group of mice. On a control, ketogenic, and low-carbohydrate, high-fat (HFLC) diet. The last two diets caused a decrease in glucose and an increase in ketone bodies which increased the survival rate of animals in these groups. This study indicates that the HFLC diet is an effective alternative to KD. This is crucial for post-treatment patients who refuse or are contraindicated in following a strict ketogenic diet for the rest of their lives. The results seem interesting, and this thesis should be examined more closely (Martuscello, 2015).

In opposition to these positive results, there are numerous studies that do not support the effect of the KD diet. In a study by Zhou et al. three interventions were used in mice bearing U87-MG glioblastoma. A ketogenic diet without caloric restrictions, a ketogenic diet with caloric restrictions and a standard diet. Tumor growth was inhibited and the survival rate increased only in the group of mice with ketosis and caloric deficit. This would indicate that reducing the daily caloric intake along with ketosis is the only effective method. There was no caloric deficit-only group in this study, which does not explain the results of the study (Zhou, 2007). A 2016 study, however, where two groups of sick mice on a caloric-deficient diet were compared, and one of them was additionally put

into ketosis, no differences were found between them. The effect was neither recorded in terms of survival nor tumor growth inhibition was observed, although both the glucose levels in the subjects were low and the ketone levels were high (De Feyter, 2016). A study on mice on a ketogenic diet and those in the control group should also be cited. Both groups had statistically similar results in terms of survival and tumor growth (Maurer, 2011).

HUMAN MODEL RESEARCH

The results of human studies appear to be highly debatable. The type of diet (caloric restriction, amount of carbohydrates), the duration of the intervention, the number of patients who completed the study compared to the initial group, and the results (tumor growth, survival, quality of life, dietary side effects) should be taken into consideration.

The 2014 research was started by 20 patients and 8 completed. Patients with standard treatment were put on a ketogenic diet containing plant oils, without caloric restriction. Among 8 patients with stable ketosis, life was prolonged without tumor progression, however, it should be remembered that the study only lasted 6 weeks. Patients reported improved quality of life on a ketogenic diet. The authors concluded that the KD diet was unsuccessful when used without standard treatment (Rieger, 2014). Similar results were obtained by Champ et al. in patients after tumor resection, radiotherapy and the KD diet. The tumor did not recur within 9 months (Champ, 2014). Mention should be made of another study using perillyl alcohol (POH) and a ketogenic diet in patients with recurrent glioma. The survey completed 9 of 17 participants. After three months of treatment, partial response was observed in 77.8% of patients, stable disease in 11.1% and progressive disease in 11.1%. Compared to the control group (standard diet, n = 8), partial response was 25%, stable disease 25% and PD 50% (Santos, 2018). Strowd et al. used a modified Atkins diet (20g of carbohydrates per day) in 8 patients for 2-24 months did not achieve a significant extension of life compared to the expected. The only advantage of nutritional ketosis was the reduction of epileptic seizures among patients (Strowd, 2015). In a study by Martin-Mc Gill et al. after 3 months of KD intervention (20 grams of carbohydrates per day) and standard therapy, patients' weight decreased but their quality of life improved according to subjective judgment (Martin-Mc Gill, 2018). In a study by Martin-Mc Gill et al. in 2020, a ketogenic or ketogenic diet with MCT was introduced in 12 people diagnosed with glioblastoma. Only 4 people completed the three-month intervention, including one at MCTKD. Global Health Status (GHS) improved in patients after KD and decreased in patients after MCTKD (Martin-Mc Gill, 2020). In these studies, we know nothing about tumor progression and survival. Patients on the KD diet and standard treatment after 3 months saw a reduction in BMI, fatigue and the number of epileptic seizures, increased energy levels, physical mobility, and improved mood and cognitive functions. However, no information was provided on tumor progression (Panhans 2020). Latest research on 50 patients who were divided into two groups where re-irradiation combined with either SD (standard diet) or KD-IF (calorie-restricted ketogenic diet and intermittent fasting), 3 days of ketogenic diet (KD: 21-23 kcal/kg/d, carbohydrate intake limited to 50 g/d), followed by 3 days of fasting and again 3 days of KD. After one month, 20 patients who completed the study found that cognitive function had changed, such as progression-free survival (PFS) and overall survival (OS) with significant differences and Quality of Life unchanged (Voss, 2021). Among patients who did not receive standard treatment, only caloric reduction and nutritional ketosis did not show positive changes after 3 months. During this time, the tumor increased in size and the patient's body weight decreased (Shwartz, 2015).

CASE STUDY

In a study on a patient with glioblastoma after standard therapy, a ketogenic diet and caloric restrictions were applied. There was a 20% loss in body weight, but the final BMI was normal (20 kg/m^2). During 5 months of caloric restriction and 2 weeks of nutritional ketosis, the results of tomography did not show an increase in tumor volume, however, 10 weeks after discontinuation of diet, the tumor began to grow back (Zuccoli, 2010).

A patient with growing GBM IDH1 glioma refused standard treatment in addition to tumor resection. Before and after the procedure, she used KD. After the procedure, slow tumor

growth was noted, but the patient's survival time without chemotherapy and radiotherapy was 80 months at the time of publication of the case study (Seyfried, 2021).

In another study on a 38-year-old man diagnosed with glioblastoma, apart from a ketogenic diet, standard therapies (resection, chemo-radiotherapy), vitamin and mineral supplementation, treatments in a hyperbaric chamber and numerous additional medications were also used. After 9 months, the seizures weakened and the tumor shrinked. However, the authors cannot determine which intervention brought the best results (Elsakka 2018).

DISCUSSION

The results of human studies appear to be inconclusive. The research is completed by a small percentage of patients, which makes the analysis even more difficult. This is due to the lack of effects, poor diet tolerance, or complications that may be associated with it. In many studies there is no control group, dietary assumptions often differ, and the state of ketosis results in a reduction in calories, which may also influence the development of the disease. Many study authors admit that KD intervention without standard treatment is usually of no avail. It should be noted that scientists emphasize that the best results of the KD diet occur when combined with other therapeutic methods such as tumor resection, chemotherapy, radiotherapy, vitamin and mineral supplementation, treatments in a hyperbaric chamber or additional drug support (Elsakka, 2018). The benefits of KD in cancer and glioblastoma are very important. One of them is reducing the tumor, stopping its growth, and thus increasing the survival time (Rieger, 2014; Champ, 2014; Santos, 2018). Other patient reported benefits include a reduction in fatigue and epileptic seizure frequency (Strowd, 2015; Martin-Mc Gill, 2018, Panhans, 2020). Despite many positive reports, one cannot forget about the problems and threats that KD brings. During nutritional ketosis, reported side effects were hypoglycaemia, hypernatraemia, hypocalcaemia, gastrointestinal complaints (diarrhea, nausea, vomiting, dyspepsia), and dry mouth (Martin, 2020). Enother side effects of the ketogenic diet is "keto-flu". It occurs during the first week of diet as the body adapts to the new composition of nutrients. It manifests with gastrointestinal symptoms, headache and muscle cramps which are results of a loss of fluid and electrolytes as well as metabolic acidosis (Bostock, 2020). All these negative aspects mean that few people are able to stick to the ketogenic diet. In a study by Kossoff et al. the diet was started by 30 adult patients with incurable epilepsy who were treated with the Atkins diet variant (15 g of carbohydrates/day). After a month, there were 26, after 3 months, 20, 6 months lasted 14. People who stopped the diet reported lack of effectiveness (9 people), 6 considered the diet difficult to implement, and one 1 did not start the diet despite registration (Kossoff, 2008). In another study, patients who gave up on diets indicated reasons such as "too restrictive", "inconvenient" (Panhans, 2020).

Caloric restrictions in dietary intervention often occur unintentionally due to decreased appetite on a high-fat diet (Pondel, 2020). In studies, the caloric deficit itself is often a factor supporting the treatment of glioblastoma (Zuccoli, 2010). It remains to be considered whether the factor affecting the health of patients with glioblastoma during the intervention is dietary ketosis, a caloric deficit, or a combination of both? The caloric deficit is often a disadvantage in patients who are of normal weight or are already in the state of cancer cachexia. It is known that malnutrition is a very common cause of death among cancer patients. Cancer cachexia is characterized by loss of body weight with specific losses of skeletal muscle and adipose tissue. It negatively affects quality of life, physical, emotional and social well-being and responsiveness to chemotherapy. Adequate nutritional support remains a mainstay of cachexia therapy (Baracos, 2018; Sadeghi, 2018).

The long-term effects of LCH diets are still unknown. It is known that due to the predominance of saturated fatty acids, it may worsen the results of the lipid profile (increase in LDL, VLDL, non-HDL fractions) and promote cardiovascular diseases (Pondel 2020). In research by Rosenbaum et al. the KD was associated with increased cholesterol and inflammatory markers after 4 weeks (Rosenbaum, 2019) (O'Neil, 2020). Due to the elimination of numerous products on the KD diet, possible deficiencies may occur. It is recommended to enrich the ketogenic diet with supplementation of water-soluble vitamins such as: vitamin B_1, B_2, B_6, niacin, folic acid, biotin, pantothenic acid, zinc, selenium, calcium, carnitine and omega-3 acids (Pondel, 2020). Most of these ingredients which

may be deficient during the KD diet actively support the immune system in anti-cancer immune responsiveness (Soldati, 2018). The diet that, according to many studies, shows the most effective anti-cancer properties is the Mediterranean diet. It is rich in products such as whole grains (about 50-60% of the total caloric intake), vegetables, fruit and legumes, which are mostly not allowed on ketogenic diet. In the long term, the lack of these products and the ingredients they contain may paradoxically contribute to cancer recurrence (Divella, 2020). Lack of consumption of the right amount of fiber and prebiotics may adversely affect the condition of the microflora of patients during the KD diet, at the same time favoring inflammatory and neoplastic processes (Soldati, 2018). Medicine still needs to search for new therapeutic combinations that include dietary metabolic regulation with conventional therapies. Perhaps this will avoid the side effects of long-term chemotherapy or multiple irradiations. (Strowd, 2015).

CONCLUSION

Despite numerous studies related to the ketogenic diet in the treatment of cancer, inclu-ing glioblastoma, there is still no clear position. There are more and more questions that need to be answered before KD is proposed as one of the treatments for cancer. The contraindications for the use of KD or the criteria for when the diet should be discontinued should be clearly defined. This could be too low body weight, hypoglycaemia or gastrointestinal side effects. It is worth defining a dietary management schedule if it is decided to be used one of the LCH diets. Uniform recommendations must be made for the diet to be most effective while minimizing side effects. Accurate education of patients who choose this type of therapy is essential. The first time of adaptation to KD should take place in the hospital ward so that the impact of "keto-flu" or other side effects on the patient's well-being can be minimized.

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Changes in skin physiology in patients during cancer treatment

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ABSTRACT

Invasive cancers pose a major challenge for the public health sector around the world. The Key methods of treatment, which currently include surgery, chemotherapy and radiotherapy, always negatively affect the patient's skin to a greater or lesser extent, disrupting its proper metabolism. For the most part, these changes are temporary and reversible, but the lack of knowledge about the side effects of therapy can be extremely surprising for the patient and is associated with severe stress.

The aim of this work is to present skin physiology in oncologically treated patients, with a particular emphasis on the characteristics of radiation-induced reactions and dermatitis resulting from chemotherapy.

Chemotherapeutic drugs are based on toxic compounds, the purpose of which is to inhibit the rapid proli-feration of cancerous cells. Unfortunately, their action is not selective and, at the same time, other normal cells with intensive cell division are also damaged, including cells of the bone marrow, gastrointestinal tract, as well as cells of the epidermis and skin appendages, i.e. hair and nails.

In radiotherapy of cancerous lesions, the purpose of the emitted ionizing radiation is to act as precisely as possible on the area affected by the growth process. The skin, therefore, is an organ damaged every time, regardless of the location of the cancerous tumour or the administered method of radiotherapy. Depending on onset of a radiation-induced reaction, local skin lesions may be divided into early (acute) and late. Radiation-induced dermatitis is a common problem – it affects 80-95% of patients treated with radiotherapy.

The characteristics of skin disorders as a consequence of oncological therapy, still remains a niche topic. Therefore, the organization of current information on the topic became the authors' motivation to write the article.

INTRODUCTION

Invasive cancers pose a major challenge for the public health sector around the world.

For example, it is estimated that every day in the United States, the number of new, registered cases may be close to 4800 (Siegiel, 2019). Cancer formation is the endpoint of a multistep process called carcinogenesis. As a result of a mutation, a dysregulated, uncontrolled cellular proliferation occurs, the final effect of which is the formation of a tumour (Presta, 2020).

The worrying, growing number of diagnosed cancer diseases is the result of multiple factors, which include a decrease in physical activity, obesity, smoking, alcohol intake, chronic exposure to selected chemical agents or a change in typical reproductive patterns. The issue of increased life expectancy for both women and men is also crucial (Torre, 2015). It is extremely difficult today to completely eliminate all potential carcinogenic factors. Invasive cancers are currently one of the leading causes of death in the human population (Wang, 2018).

In the case of suspicion of cancer, it is very important to have a quick and thorough diagnosis, enabling the assessment of the stage of the disease, and thus the application of appropriate therapy. Hormone and biological therapies are used to treat oncological patients, but surgical procedures, chemotherapy and radiotherapy remain the key treatments, applied as standalone or combined methods (Wang, 2018).

Once diagnosed with cancer, it is very important for the patient to obtain full information regarding the proposed treatment methods and any consequences associated with them. Skin is a large human organ, the physiology of which undergoes significant changes as a result of oncological treatment. The skin, especially in the face and neck area, is an element conditioning the aesthetics of appearance. Unfortunately, any form of treatment has a negative

effect on both the epidermis and the dermis. For the most part, these changes are temporary and reversible, but the lack of knowledge about the side effects of therapy can be extremely surprising for the patient and is associated with severe stress. The quality of life for oncological patients, defined as the perception of a patient's own physical, mental and social health, becomes significantly reduced (Mokhatri-Hesari, 2020). This is due to the deterioration of the general condition, and is also associated with a change in appearance, which is often very drastic. Therefore, making the patients aware that most of the side effects disappear after the therapy, gives strength to the patients and thus motivates them to fight the disease.

SEARCH STRATEGY AND SELECTION CRITERIA

The aim of this work is to present skin physiology in oncologically treated patients, with a particular emphasis on the characteristics of radiation-induced reactions and dermatitis resulting from chemotherapy.

The epidermis, dermis, as well as skin appendages undergo significant changes, but the characteristics of skin disorders as a consequence of oncological therapy, still remains a niche topic. Therefore, the organization of current information on the topic became the authors' motivation to write the article.

The study refers to international institutions dealing with the issue of cancer (the International Agency for Research on Cancer – IARC, the World Health Organization – WHO, the European Cancer Organisation) and uses recognized medical databases to verify the latest publications (PubMed, Cochrane Database of Systematic Reviews, Google Scholar). All databases were searched using a combination of keywords: quality of life oncology, cancer statistics, cancer therapy, the hand-foot syndrome, toxic erythema chemotherapy, toxic side effect chemotherapy skin, chemotherapeutic hair, nail disorders chemotherapeutic, radiotherapy, ionizing radiation, and radiation induced dermatitis.

Eligibility criteria for inclusion were review articles and original articles on invasive cancers and oncological therapy-induced dermatitis. The work is based on the latest and most relevant data.

REVIEW

CHEMOTHERAPY

Chemotherapy (CHT) acts as the leading method of the systemic treatment of malignant tumours, which means that it affects the patient's entire body. It is effective both in the case of a focal lesion and in the diagnosis of metastases, since the drugs applied are able to reach every organ of the body via the bloodstream. Chemotherapeutic drugs are based on toxic compounds, the purpose of which is to inhibit the rapid proliferation of cancerous cells. Unfortunately, their action is not selective and, at the same time, other normal cells with intensive cell division are also damaged, including cells of the bone marrow, gastrointestinal tract, as well as cells of the epidermis and skin appendages, i.e. hair and nails (Pérez-Herrero, 2015).

Since the application of the first drugs approved by the Food and Drug Administration (FDA) for the treatment of solid tumours and haematological cancers (such as methotrexate, nitrogen mustards, antifolate drugs) in the 1940s and 1950s, chemotherapy has significantly evolved. The treatment is becoming more and more effective, but despite medical advances, side effects accompanying the treatment, like deviations in blood counts, vomiting, changes in the condition and appearance of the skin, hair, eyelash, eyebrow loss or significant fragility of nails are still a big problem for patients (Pérez-Herrero, 2015; Chabner, 2005).

Antineoplastic drugs are a wide group of substances with anticancer activity. The mechanism of their action consists in blocking the cell cycle and triggering programmed cell death – apoptosis in order to eliminate cancer cells. Generally, a multidrug chemotherapy is used. Only in justified cases, it is possible to use a single drug. For effective therapy, antineoplastic agents of different classes are combined to enhance the cytostatic effect. Chemotherapy can be applied to patients both before (neoadjuvant CHT) and after surgery (adjuvant CHT). It is often given in combination with radiotherapy. Antineoplastic drugs can be divided into two groups, i.e. the cell cycle phase specific (cell cycle phase dependent) and the cell cycle phase non-specific (or cell cycle phase independent). The cell cycle phase specific drugs have an impact on cells that are in a specific phase of the cell cycle. They can act on cells in phase S –

methotrexate, 5-fluorouracil or cells in phase M – vinblastine, vincristine. In addition, there are antineoplastics acting on cells in the G1 phase – corticosteroids, or the G2 phase – bleomycin. On the other hand, drugs referred to as cell cycle phase independent, have an impact on dividing cells, regardless of the phase of the cycle. This group of drugs includes alkylating drugs and antibiotics with anticancer activity. Chemotherapy is most effective in the treatment of early-stage cancer (Pérez-Herrero, 2015; Hanahan, 2011; Ingham, 2017; Mutsuga, 2002).

EFFECTS OF CHEMOTHERAPY AND SYSTEMIC TREATMENT ON SKIN PHYSIOLOGY

Antineoplastic drugs significantly contribute to the impairment of the condition and appearance of the skin and its appendages. The severity and course of side effects of chemotherapy depend on the type, dosage and combination of drugs administered, as well as the individual reaction of the patient's body. Although complications in most cases do not pose a threat to life, they notably worsen its quality and limit the patients' daily activities.

Antineoplastic drugs cause dilution of the epidermis, including the stratum corneum, which affects the weakening of the barrier functions of the skin. Damage to the hydrolipid film, which is the natural protective barrier of the skin, is visible. As a result, transepidermal water loss (TEWL) increases. The skin becomes dehydrated, and thus dry, red and scaly. These symptoms are usually accompanied by persistent itching. Damage to the protective barrier makes the skin susceptible to bacterial, viral and fungal infections. The risk of infection is intensified by the fact that the patient's immune system during chemotherapy is weakened.

Metabolites of the drugs taken are partially released through the sweat glands, therefore the areas rich in eccrine glands are particularly vulnerable to side effects (Yosipovitch, 2019; Jennings, 2020; Owczarek, 2017).

In patients treated with chemotherapy, the hand-foot syndrome – HFS, otherwise known as hand-foot skin reaction (HFSR), palmoplantar dysesthesia, or acral erythema is very often observed. HFS is one of the most frequently diagnosed dermatological complications in the course of oncology treatment. Symptoms can occur from 24 hours up to 10 months from the beginning of therapy, which correlates with the type of medication administered. It is characterized by the formation of erythematous lesions on inner surfaces of the hands and soles of the feet. Usually, the first symptoms are a feeling of warmth in the skin and erythematous lesions. Gradually, there is tingling, anesthetizing, paraesthesia and associated problems with holding objects and even walking. These symptoms may be due to neuropathy of fine nerve fibres. In the course of HFS, swelling and blisters on erythematous medium may occur. On the skin, foci of hyperkeratosis are formed together with strong exfoliation, even bleeding. Skin lesions may be accompanied by pain that limits the performance of instrumental activities. The drugs most commonly causing HFS include 5-fluorouracil – 5-FU, pegylated liposomal doxorubicin (PLD), docetaxel, capecitabine. The pathogenesis of HFS is not fully discovered. However, it is believed that the high concentration of eccrine glands in the hands and feet causes high exposure of the skin to chemotherapeutic agents in these areas, due to the release of drug metabolites through the sweat glands (Chidharla, 2021; Kwakman, 2020). The following table (Table 1) shows the severity of HFS symptoms according to the National Cancer Institute – Common Terminology Criteria for Adverse Events (NCI-CTCAE).

Table 1. Symptom severity classification in the hand-foot syndrome according to the NCI-CTCAE (Chidharla, 2021)

HFS advancement level according to the NCI-CTCAE v. 4.0	characteristics of lesions
grade 1	minimal skin changes or dermatitis (e.g. erythema or oedema) without pain
grade 2	skin changes (e.g. peeling, blisters, bleeding, or hyperkeratosis) with pain leading to limitation of instrumental activities of daily living (ADL)
grade 3	severe skin changes (e.g. peeling, blisters, bleeding, oedema, or hyperkeratosis) with pain, limiting basic self-care ADL

In a group of patients treated with inhibitors of epidermal growth factor receptor inhibitor (EGFRI) and mitogen-activated protein (MAP) kinase kinase inhibitor (MEKI), acneiform rash is the most commonly diagnosed. These changes usually develop within 2-4 weeks of treatment. The clinical picture consists of papules and pustules, which may be accompanied by itching and even pain. Sometimes spontaneous bleeding from the lesions occurs, which significantly worsens the quality of patients' lives. The acneiform rash is located mainly on the face, especially in the middle part, in the behind-the-ear area, on the neck and in the upper part of the chest, i.e. in the areas rich in sebaceous glands, which is why it is referred to as "acneiform". The EGFR inhibitors disrupt the natural balance between proliferation and differentiation of keratinocytes. An inflammatory reaction develops. The incision of hair follicles by T lymphocytes is observed. Sometimes the histological image reveals damage to the hair apparatus, the influx of neutrophils and even abnormal structure of sweat glands (Lacouture, 2018; Lacouture, 2006; Wu, 2011; Kowalska, 2016).

Hair follicles are one of the main structures that are damaged during treatment with antineoplastic drugs. Anticancer drugs damage cells of the hair matrix, which are characterized by high proliferation recorded in the anagen phase. Hair follicles are extremely sensitive to the toxic effects of drugs (Trüeb, 2010). After 2-4 weeks of treatment, the separation of the hair fibre from the hair bulb begins. Initially, soreness of the scalp may be felt. The process of hair loss depends significantly on the type of drugs administered. For example, the use of anti-microtubule agents in the treatment causes baldness in 80% of patients, while topoisomerase inhibitors lead to alopecia in 60-100% of patients (Rossi, 2017).

Some antineoplastic drugs do not cause complete hair loss. Hair can only become thinner, dry, brittle, and less thick. Also, the dose of an antineoplastic drug has a significant effect on the severity and course of alopecia. Polychemotherapy is associated with higher incidences compared to monotherapy. With a low dose of chemotherapeutic agents, hair loss may be slower or less intense. The individual predisposition of the oncological patient is also an important factor. Alopecia may occur suddenly or hair may fall out gradually. Different shedding patterns are observed, both dystrophic anagen effluvium and telogen effluvium. It should be noted that hair loss affects not only the hairy scalp, but the whole body. There is a loss of eyelashes, eyebrows, facial hair, hair on the limbs, armpits and intimate areas. Hair begins to grow back a few months following the end of antineoplastic drug therapy, and full hair regrowth usually occurs up to a year after the end of treatment. The new hair may differ in colour, structure and thickness. It is usually darker and more curly than before (Trüeb, 2010; Heidary, 2008; Rossi, 2017).

The side effects of chemotherapy also appear in the nail area. Changes can take different forms. They concern the appearance of the nail plate, the slower growth or separating of the plate from the nail bed (onycholysis). Nails become tender and can hurt. They are prone to mechanical injuries. Onychomycosis or bacterial infections are more common, caused, for example, by Staphylococcus aureus, and leading to the development of abscesses under nails or in the nail area. Patients' nails tend to split. Discoloration may appear on their surface as a consequence of the deposition of drug metabolites in the plate or nail bed. Discolorations are usually black or blue in colour. In some patients, vertical or horizontal furrows are visible, arranged parallel to each other. Antineoplastic drugs also have a significant impact on slowing down the growth of the nail plate (Kowalska, 2016; Wasner, 2001; Roh, 2007).

RADIOTHERAPY

Radiotherapy (RTH) is an important aspect of invasive cancer treatment. It is based on the use of ionizing radiation to damage abnormal cells. The effect of ionizing radiation on a living organism may be direct and indirect. As a consequence of photon absorption, the centre is ionized and electrons detach, which damages the most sensitive elements of the cell (DNA, cell membranes). Such a mechanism of damage to living matter is referred to as direct mechanism. Definitely, more damage to cellular structures is induced by the activity of reactive oxygen species (ROS) formed as a result of the water radiolysis process, in the so-called indirect mechanism. Reactive oxygen species cause about 75% of radiation-induced damage (Mondini, 2020; Ryan, 2012; Mazurek, 2018).

Radiotherapy is generally used in order to fully cure a sick person (radical radiotherapy). How-

ever, in a situation of significant advancement of the disease, it is used to maximize the patient's life or improve their comfort. It is then referred to as palliative radiotherapy. RTH can be both neoadjuvant – preceding the main treatment, which is most often surgery, and adjuvant – supplementing the basic treatment. In many cases, it is also a complement to chemotherapy. This method of treatment is successfully used in the treatment of many invasive cancers, including the treatment of malignant head and neck cancers, breast cancer, malignant cancers of the reproductive system in women or prostate in men (Rai, 2015; Murthy, 2016).

THE EFFECT OF RADIOTHERAPY ON SKIN PHYSIOLOGY

In RTH of cancerous lesions, the purpose of the emitted ionizing radiation is to act as precisely as possible on the area affected by the growth process. In the therapeutic field, however, there is always a fraction of healthy tissues. Undesirable effects include radiation-induced reac-+tions, which are the response of the epidermis and dermis to ionizing radiation. The intensity of radiation-induced reactions depends on the patient's age, general health, possible concurrent diseases, the advancement stage of the disease, the histological image of the tumour, the dose fractionation scheme used or the use of simultaneous chemotherapy (Ryan, 2012; Mazurek, 2018).

The skin, therefore, is an organ damaged every time, regardless of the location of the cancerous tumour or the administered method of radiotherapy. Depending on onset of a radiation-induced reaction, local skin lesions may be divided into early (acute) and late. Early radiation-induced reactions of the skin develop during radiation therapy and up to 6 months after its completion, and are mostly temporary. They usually cover the area directly exposed to radiation. Initially, these are epidermal lesions, because the epidermis reacts faster. Subsequent fractions of radiation gradually damage the dermis as well. The first noticeable symptom is erythema of a transient nature, caused by an increased activity of pro-inflammatory cytokines, such as the interleukin-1 (Il-1) and Il-6, the tumour necrosis factor α (TNF-α) and the transforming growth factor β β (TGF-β). The erythema becomes pale pink to bright or smoky pink in colour. Overstimulation of pigment cells takes place as well. As a result of damage to the keratinocytes of the stratum basale, dry exfoliation of the epidermis is observed, and at a later stage, there is moist exfoliation, accompanied by serous exudate. As a result of the destruction of all cells of the stratum basale, the dermis becomes exposed. Skin irritation is characteristic of acute radiation-induced dermatitis, which is accompanied by a feeling of warmth and itching, sometimes there is also pain. The activity of sweat and sebaceous glands is noticeably reduced, which is manifested by dryness of the skin. Dystrophic alopecia is observed in radiated areas. Permanent hair loss is not to be excluded when high doses of ionizing radiation are applied (Ryan, 2012; Stone, 2003; Chan, 2014).

Late radiation-induced dermatitis develops from 6 months to several years after the completion of radiation treatment. Disorders in the functions of mature fibroblasts are observed. Fibroblasts are cells of the connective tissue and are responsible for the production of collagen and elastin. As a consequence of the ionizing radiation, a reduced number of fibroblasts is observed, which begin to produce a greater amount of collagen fibres, with irregular arrangements. The result of these changes are manifested in skin abnormalities such as loss of elasticity, the presence of skin fibrosis, increased hardness, as well as swelling. As a result of damage to the vascular endothelium, there is a permanent expansion of blood vessels – visible on the skin in the form of telangiectasia. Nerve fibres also become damaged, which translates into sensory disturbances. Atrophic changes may also develop, and in extreme cases, dermal necrosis. The sebaceous and sweat glands undergo atrophy. In the case of late radiation-induced reactions, the dose of absorbed radiation is of great importance. The higher the dose, the faster the reaction can be visible. It should be understood that the appearance of a late reaction is essentially irreversible. The most severe consequence of radiation therapy is the risk of induction of secondary cancers (Chan, 2014; de Andrade, 2012; Iacovelli, 2020).

Radiation-induced dermatitis affects 8-95% of patients treated with radiation therapy (Ryan, 2012; de Andrade 2012) with the head and neck region, the breasts and perineal area being particularly vulnerable to acute radiation-induced reactions. It is worth emphasizing that radiation therapy does not only affect the area subjected to radiation, but to some extent, it has

an impact on the whole body. In addition to discomfort of the skin or mucous membranes, the patients also report general symptoms such as malaise, drowsiness or weakness (Iacovelli, 2020).

In order to correctly assess the severity of the local radiation-induced dermatitis, specific scales are applied. One of the most commonly used is the RTOG/EORTC (Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer) scale. It allows for the assessment of early and late radiation-induced dermatitis in the degree of severity from 0 to 5. However, practice has shown that this scale has some limitations and also does not take into account the symptoms reported by patients (Mondini, 2020). Another scale, the Dische scale, focuses on damage to the mucous membranes and takes into account clinical disorders (pain, problems with swallowing) in addition to clinical symptoms (erythema, epitheliolysis, oedema). To assess late changes, the LENT-SOMA (Late Effect Normal Tissue Task Force – Subjective, Objective, Management, Analytic) scale is used. This scale determines the degree of skin fibrosis, the surface of telangiectasia and the severity of skin discoloration. In addition to the above, the NCI-CTCAE (National Cancer Institute – Common Terminology for Adverse Events) scale is also used. It is a 5-degree scale, the first degree of which means weak erythema or dry exfoliation, and the last means death. There is also the RISRAS (Radiation-Induced Skin Reaction Assessment Scale) that details and objectively assesses both dermic lesions and the subjective sensations of the patient, which are equally important (Stryczyńska, 2011; Raza, 2012; Kumaran, 2014).

DISCUSSION

Oncological therapy is directed towards rapidly dividing cancer cells, but there is always damage to normal cells, characterized by a high proliferative index. The skin is classified as an organ whose physiology changes significantly as a result of therapy, which is confirmed by researchers studying the impact of individual therapeutic methods used on the patient's skin in oncology (Choi, 2014; Macdonald, 2015; Anforth, 2015).

The key methods of treatment, which currently include surgery, chemotherapy and radiotherapy, always negatively affect the patient's skin to a greater or lesser extent, disrupting its proper metabolism.

For many patients, a postoperative scar is a significant problem, but it should be high-lighted that any aesthetic procedures to reduce it can be carried out after a sufficiently long time following the procedure and after obtaining permission from the oncologist. Apart from the dermatitis that accompanies chemotherapy, one of the most difficult adverse events connected with treatment, especially for women, is alopecia. On average, 65% of patients who have received treatment (de Barros Silva, 2020) are affected by this problem. 47% of patients perceive alopecia as the most traumatic aspect of chemotherapy (Trüeb, 2010). There are cases when the decision to start treatment is delayed or even declined for fear of hair loss.

One of the ways to reduce chemotherapy-induced alopecia (CIA) is to use scalp cooling during chemotherapy by using special machines ("caps"), e.g. Paxman, Dignitana. In Brazil, three scalp cooling devices to be used during chemotherapy have received approval from the Brazilian Health Regulatory Agency (Agência Nacional de Vigilância Sanitária) (de Barros Silva, 2020). However, access to this method is not common. It also seems that the safety of using this method and the absolute elimination of the possible negative impact of cooling on the effectiveness of treatment requires further study.

Radiation therapy, in addition to its positive aspects, is also the cause of bothersome and unesthetic skin lesions in the form of radiation-induced dermatitis. Research in the field of psychodermatology indicates that appearance has an extremely important impact on self-acceptance, satisfaction and self-esteem, which take on particular importance in the case of cancer.

Although many argue that in the face of cancer the quality of life seems to be a secondary issue, it should be remembered that stress and depression lead to a worse prognosis, although this mechanism has not been fully explained (Lang-Rollin, 2018). Thus, a thorough conversation with the patient, discussing the negative consequences of therapy, largely manifested by the deterioration of the function and appearance of the skin and its appendages, are of colossal importance. Making the patient aware that, to a large extent, these are only temporary dysfunctions and will make the patient prepared for the

side effects of the treatment and certainly cope with them better. It should also be emphasized that currently, there are many pharmacological and cosmetology methods that largely alleviate the skin-related side effects of oncological therapy. The developing field of oncology aesthetics has a positive effect not only on the appearance of the patients, but it also improves their well-being, which gives them strength to conquer the disease.

SHORT CONCLUSIONS

1. Chemotherapy and radiotherapy negatively affect the appearance and functioning of the skin and its appendages. Complications of oncological treatment significantly worsen the quality of life of patients.
2. Cytostatic drugs weaken the barrier functions of the skin, contribute to an increasing of the transepidermal escape of water, and thus to skin dehydration. The skin becomes susceptible to bacterial, viral and fungal infections.
3. The hand-foot syndrome (HFS) is one of the most frequently diagnosed dermatological complications in the course of oncological treatment.
4. Radiation-induced dermatitis is a common problem – it affects 80-95% of patients treated with radiotherapy.
5. Taking into account the time criterion, there are early radiation-induced reactions, manifested by a change in the anatomy and physiology of the epidermis and late radiation-induced reactions, in the course of which significant deviations are also observed not only in the epidermis, but also in the dermis.
6. One of the most difficult experiences associated with oncological treatment is alopecia, which affects on average 65% of patients.
7. Proper skin care during and after oncological therapy can contribute to the reduction of side effects and accelerate skin healing and its return to normal condition.

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