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Molecular Prognostic and Predictive Markers in Triple - Negative Breast Cancer

Marketa Kolečková, Katherine Vomacková and Zdeněk Kolar

Abstract

Triple-negative breast cancer (TNBC) is defined as a molecular subtype of breast cancer that lacks expression of hormone receptors (oestrogen and progesterone receptor) and HER2/neu/ErbB2 protein. It accounts for 15–20% of all invasive breast cancers. The occurrence of TNBC is often associated with younger age at the time of diagnosis and pre-menopausal status, early onset of menarche, higher body mass index (BMI) in the pre-menopausal period, race and ethnicity (African, Hispanic) and the presence of germline mutation in the BRCA1/2 genes or somatic mutation in the TP53 or PTEN genes. TNBCs are specific in its aggressive biological behaviour, shorter interval to disease progression and more frequent relapse within five years (19 to 40 months). The most of TNBCs are represented by high-grade invasive carcinomas of no special type (NST) with high proliferation index measured by Ki-67 nuclear expression, followed by metaplastic carcinomas, secretory carcinomas, and adenoid cystic carcinomas. Genetical and morphological heterogeneity inside TNBC is responsible for the higher frequency of primary and secondary resistance to systemic therapy. The scope of this chapter is to summarise the potential therapeutic agents involved in regulation of cell proliferation, migration, angiogenesis, apoptosis, gene expression and DNA damage or immune response. The insight into this issue is essential for the setting of the optimal chemotherapy regimen and targeted therapeutic strategy.

Keywords: Triple-negative breast cancer, prognosis, prediction, molecular target

1. Introduction

Triple - negative breast cancer (TNBC) represents a morphologically and genetically heterogeneous molecular subtype of breast cancer lacking the expression of hormone receptors (oestrogen and progesterone receptor) and HER2/neu/ErbB2 protein. It accounts for 15–20% of all cases [1]. The occurrence of TNBC is often associated with younger age at the time of diagnosis and pre-menopausal status, early onset of menarche, higher body mass index (BMI) in the pre-menopausal period, race and ethnicity (African, Hispanic) and the presence of germline mutation in the BRCA1/2 genes or somatic mutation in the TP53 or PTEN genes [2, 3]. In addition, for BRCA1/2 mutant gene carriers, the risk of developing TNBC multiplies after therapeutic exposure to ionising radiation. Other genetic alterations include mutations in the RB1, NF1, ERBB3, ERBB4, ALK and EGFR genes, changes in the

NOTCH1/2, *MAST1/2* gene copy number or *MAGI3* - *AKT3* gene fusion. The gain on chromosomes 1q, 8q, 10q and the loss on chromosomes 5q and 8p were also demonstrated.

From a clinical point of view, TNBC is specific in its aggressive biological behaviour, shorter interval to disease progression and more frequent relapse within five years (19 to 40 months vs. 35 to 65 months) [4]. The median overall survival (OS) for metastatic TNBC is reported to be 9 to 12 months [5]. Due to these tumour characteristics, chemotherapy is often indicated already during the initial phase of treatment. Heterogeneity inside TNBC is responsible for the higher frequency of primary and secondary resistance to treatment [6]. The current research trends therefore focus on finding the new potentially therapeutically manageable molecules, which could significantly help to decrease the risk of metastasis development and disease recurrence.

Compared to other molecular subtypes, TNBCs differ in their high degree of gene instability. Based on the gene expression profiling, TNBC can be subclassified into several distinct molecular subtypes. Lehmann et al. represent one of the first research groups using this approach in practical diagnostics [7, 8]. Since then, a couple of classification schemes have been introduced; see **Table 1** [9–13].

The essential clue for effective breast cancer management is comprehensive evaluation of number of prognostic and predictive molecular indicators. While prognostic factors correlate with patient survival, predictive factors provide

Authors	TNBC subtype	Basic molecular characteristics
Ma et al. [9]	BL	Increased CK5/6, EGFR expression
	LAR	Increased AR expression
	“Claudin - low”	CD44+/CD24- immunophenotype
		Decreased claudin 3, 4, 7 expression
Lehmann et al. [7]	BL1	Increased Ki-67 expression
	BL2	Increased CD10, p63 expression
	LAR	Increased AR expression
		Aberrant <i>FOXA1</i> , <i>KRT18</i> , <i>XBP1</i> gene activation
	M	Aberrant regulation of Wnt, ALK, TGF-β
	MSL	Aberrant regulation of Rho, ALK, TGF-β, Wnt/β-catenin, ERK1/2, EGFR, PDGF, PI3K
	IM	Aberrant regulation of NFκB, TNF, JAK/STAT
Burststein et al. [10]	LAR	Increased AR, MUC1 expression
		Aberrant <i>PIK3CA</i> , <i>AKT1</i> , <i>CDH1</i> gene activation
	M	Increased PDGF-A, c-Kit expression
	BLIA	Aberrant regulation of STAT
		Presence of B /T/NK immune cells
	BLIS	Aberrant regulation of VTCN1
Jézéquel et al. [11]	BL	Immune cells -, TAM – like cells +
	LAR	Increased AR expression Aberrant <i>FOXA1</i> , <i>KRT18</i> , <i>XBP1</i> gene activation
	“BL - enriched”	Immune cells +, TAM – like cells -

Authors	TNBC subtype	Basic molecular characteristics
Ahn et al. [12]	BL	Aberrant <i>ATR</i> , <i>BRCA1/2</i> , etc. gene activation
	M	<i>PIK3CA</i> gene mutation, <i>PTEN</i> gene inactivation
		Aberrant regulation of PI3K / AKT
	IM	Aberrant regulation of NFkB, TNF, JAK/STAT, VTCN1, presence of B/T/NK immune cells
Zeng et al. [13]	LAR	Increased AR expression Aberrant <i>FOXA1</i> , <i>KRT18</i> , <i>XBP1</i> gene activation
	BL	Increased CK5/6, EGFR expression
	NBL	Absence of CK5/6, EGFR expression

BL = basal – like; *BL1/2* = basal – like 1/2; *LAR* = luminal androgen receptor; *M* = mesenchymal; *MSL* = mesenchymal stem – like; *IM* = immunomodulatory; *BLIA* = basal-like immune-activated; *BLIS* = basal-like immune-suppressed; *NBL* = normal breast - like.

Table 1.
History of proposed TNBC classification systems.

information on the response to a specific therapy. The all prognostic clinicopathological characteristics such as patient age at the time of diagnosis, clinical and pathological tumour stage, tumour type with detailed tumour morphology analysis including the intensity of tumour infiltrating lymphocytes (TILs), tumour grade, occurrence and extent of in situ carcinoma and family history of breast cancer should be taken into account.

The most of TNBCs are represented by high-grade invasive carcinomas of no special type (NST) with high proliferation index measured by Ki-67 nuclear expression, followed by metaplastic carcinomas, secretory carcinomas, and adenoid cystic carcinomas [14]. The morphological pattern of invasive carcinomas NST may involve medullary, lipid-rich, apocrine, pleomorphic or spindle cell areas. Carcinomas with spindle tumour cell transformation are usually related to “claudin-low” molecular subtype (CD44+ / D24– / low) and epithelial to mesenchymal transition (EMT) process [15–17]. Metaplastic breast cancers and secretory carcinomas account for 0.2 to 5%, respectively 0.02% of all breast cancers [14]. Adenoid-cystic carcinomas with typical fusion of the *MYB* - *NFIB* genes and mutations in the *EP300*, *NOTCH1*, *ER882* and *FGFR1* genes are described in 0.1 to 3.5% of breast tumours [14].

2. Molecular prognostic and predictive markers

Individual molecules involved in the process of TNBC carcinogenesis may be divided into several groups. The groups of proteins include proteins participating in mechanisms of repair of damaged DNA; proteins responsible for regulation of cell proliferation, migration, angiogenesis, programmed – cell death (apoptosis) and immune response (immune checkpoint proteins; and groups of proteins modifying gene expression (see Table 2).

2.1 Regulators in the DNA damage response

Genes and proteins involved in the repair of damaged DNA (poly (ADP-ribose) polymerase, genes with tumour suppressor function *PTEN*, *BRCA 1*, *BRCA2*, *TP53* a *RB1*) are key factors in maintaining genome integrity, ensuring that the cell cycle

Regulators in the DNA damage response	BRCA1, BRCA2, PARP, PTEN, pRb, p53
Regulators of cell migration and proliferation	EGFR, VEGFR, FGFR
Regulators of apoptosis	Fas, TRAIL, p53, Bcl-2
Regulators of gene expression	microRNA, lncRNA, circRNA, siRNA
Steroid receptors	Androgen receptor
Immune checkpoint proteins	PD - 1, PD - L1

Table 2.
Classification of molecular prognostic and predictive markers.

proceeds correctly. Deoxyribonucleic acid (DNA) may be damaged due to physical, chemical, as well as biological processes. Repair of the damaged DNA is realised by several mechanisms, including repair of mismatched bases (mismatch repair - MMR), nucleotide and base excision repair (nucleotide excision repair“ - NER; „base excision repair“ - BER) or repair of double-strand DNA breaks by homologous recombination (HR) or by non-homologous end joining NHEJ).

The enzyme family **poly (ADP-ribose) polymerase** is responsible for the transfer of the subunit (ADP) – ribose from NAD⁺ to the acceptor protein creating long, branched and negatively charged polymers of poly - ADP ribose (**Figure 1**) [18–22]. PARP-1 is the most abundant, an evolutionally highly conserved enzyme involved in the repair of damaged DNA through BER. It is composed of an NH₂-terminal domain with three „Zinc fingers“, which binds to the damaged DNA, automodification domains and C-terminal catalytic domains. The conformation change arising from the binding of PARP to the site of damaged DNA enables catalysis of the transfer of ADP-ribose from NAD⁺ to its own molecule and histone H1.

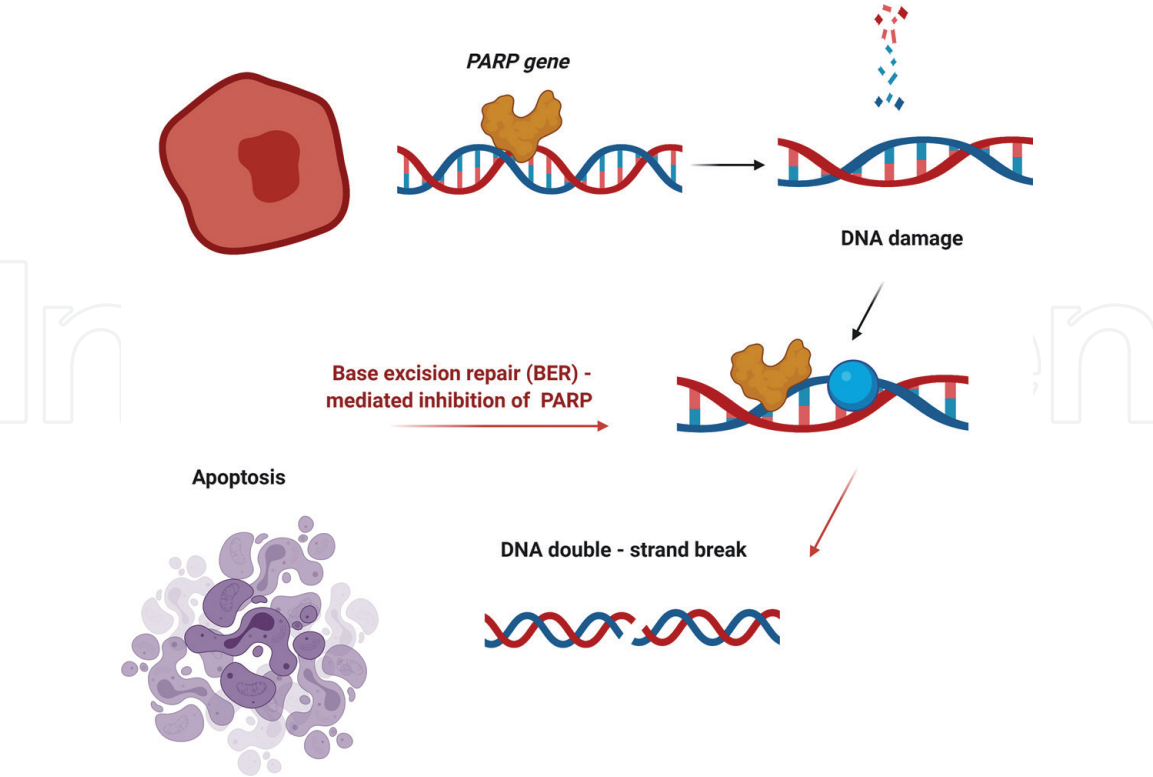


Figure 1.
Mechanism of action of PARP inhibitors (Koleckova M, www.biorender.com). Efficient single-strand breaks (SSB) repair provided by PARP is essential for the cell survival. The mechanism of action of PARP inhibitors include the suppression of this base excision repair (BER) – mediated pathway, resulting in the pathologic double-strand breaks (DSB) with homologous recombination (HR) – mediated repair and thus genome stability and the cell death.

Subsequently, degradation of the chromatin structure occurs and there is an influx of additional damaged DNA repair proteins (for example DNA ligase 3, DNA polymerase β and protein XRCC1). In patients with TNBC with a confirmed mutation in the gene *BRCA1* or *BRCA2*, PARP takes over a backup function, it inactivates the degradation of caspases and initiates apoptosis utilising so-called synthetic lethality. The direct inhibitory effect on the PI3K/AKT/mTOR and Wnt/ β -catenin signalling pathways has also been established, with corresponding changes in miRNA and serine/threonine kinase ATM expression. *PARP inhibitors* may be administered in monotherapy as well as in combination. They amplify the effect of the administered chemotherapy and/or inhibitors of molecules of the signalling pathway PI3K/AKT/mTOR, inhibitors of deacetylation of histones, CDK1, EGFR, AR, ATM or MYC. In cases treated by *olaparib* versus the chemotherapy group (capecitabine, eribulin or vinorelbine based on selection of the examining physician), the progression-free survival (PFS) was prolonged from 4.2 months to 7 months. A higher rate of therapeutic response was also discovered (59.9%). A positive finding was also observed with *talazoparib* in monotherapy, where PFS was prolonged from 5.6 months to 8.6 months, while increasing the rate of therapeutic response to 62.6%. Finally, administration of *veliparib* in combination with paclitaxel and carboplatin seems to be effective. Mechanisms leading to the development of resistance to PARP inhibitors include secondary mutations in the *BRCA1* and *BRCA2* genes, in genes coding the P-glycoprotein pump or the loss of protein 1 binding protein p53 (53BP1).

Tumour suppressor gene *PTEN* participates in the regulation of cell proliferation, migration and apoptosis under physiological conditions [23–26]. Phosphatase and tensin homologue (PTEN) represent a protein belonging to the tyrosine-phosphatase family with phosphatidyl-inositol-phosphatase activity. After binding tensin, a focal adhesion complex is created, which affects cell integrity and the transfer of intercellular as well as intracellular signals. The catalytic domain C2 is responsible for PTEN binding to the cell's phospholipid membrane and ensures Ca^{2+} dependent membrane transport of signal proteins. The resulting action of protein PTEN is the inhibition of proteins of the signalling pathway PI3K/Akt/mTOR (**Figure 2**), whose aberrant activation via activation of genes *PI3CA*, *AKT1* and *MTOR* would lead to induction of the process of cancerogenesis. Indirect activation of protein PTEN is realised by the fully functional gene *TP53* (wild – type p53 protein). Alteration in the expression of gene *PTEN* is a result of its deletion or inactivating mutation. It occurs in up to 41% of cases of TNBC and correlates with a shorter PFS and overall survival (OS). Therapeutic inhibition of aberrantly activated PI3K/Akt/mTOR of the signalling pathway is possible by administering paclitaxel in combination with ATK inhibitor *ipatasertib*. Compared with placebo, ipatasertib led to a significant prolonging of PFS - from 4.9 months to 9 months, as well as OS – from 18.4 months to 23.1 months). Similarly, effective, but with a greater number of side effects, was the combination of paclitaxel with *capivasertib* (PFS – 5.9 months; OS – 19.1 months).

Tumour suppressor genes *BRCA1* and *BRCA2* are involved in the regulatory phases S and G2 of the cell cycle [27–29]. As transcription factors, they participate in the repair mechanism of DNA single-strand breaks via HR. In cases of DNA double-strand breaks, phosphorylation of protein BRCA1 by protein kinase ATM takes place, with subsequent interaction with protein RAD51, transported with the help of protein BRCA2, and leads to repair of the damaged DNA. In case there is a loss of function of genes *BRCA1* and *BRCA2*, the *PARP* genes take over their role, inactivates caspase degradation and initiates apoptosis through mechanism of synthetic lethality. Inactivating mutations in the *BRCA1* gene were determined in 40% of cases of familial breast cancer. Autosomal dominant inheritance was found in 5–10% of patients. Confirmation of a germline mutation in the *BRCA1* gene is

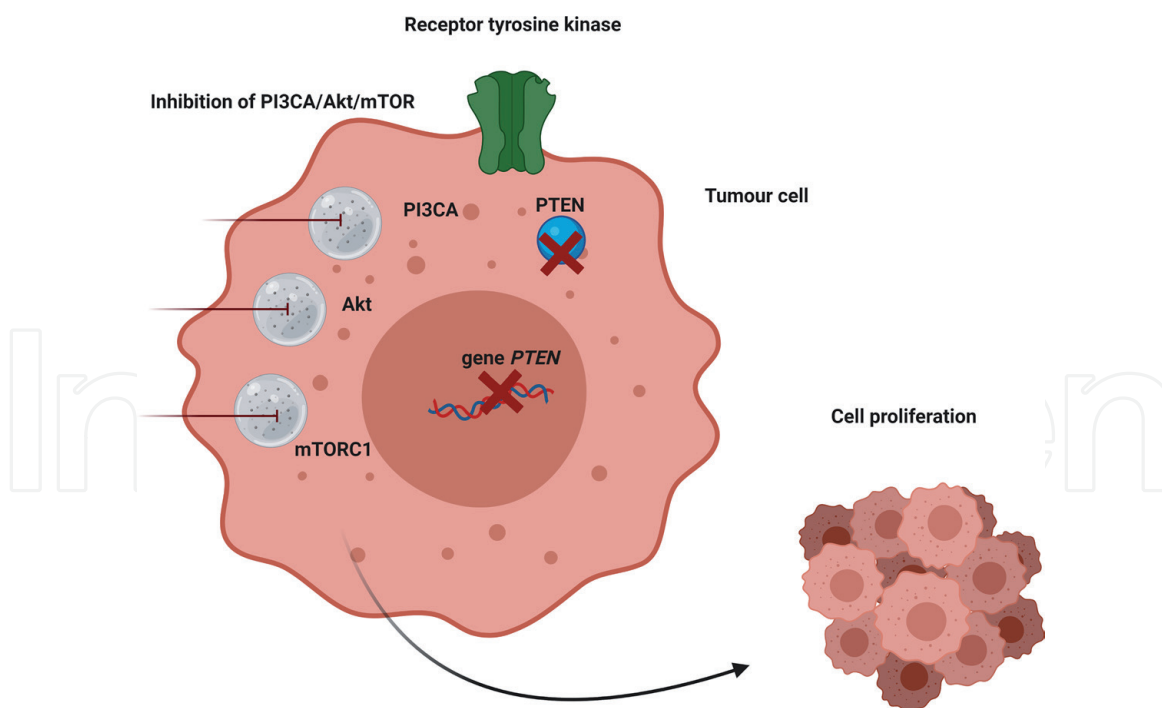


Figure 2.

Mechanism of action of PTEN protein (Koleckova M, www.biorender.com). PTEN protein (Phosphatase and tensin homologue) is essential for the regulation of intracellular levels of phosphorylation, cell migration, proliferation and survival. It plays a pivotal role in the phosphatidylinositol-3-kinase (PI3-K) pathway involved in the inhibition of proteins of the signalling pathway PI3K/Akt/mTOR. Inactivation of the PTEN tumour suppressor gene leads to the aberrant activation of PI3CA, Akt and mTORC1 genes associated with the initiation of the process of cancerogenesis.

considered to be an unfavourable prognostic marker. However, in NBC it predicts an increased therapeutic response to anthracyclines, taxanes, platinum derivatives, and in advanced disease stages, PARP inhibitors.

Protein Rb (pRb), a product of **tumour suppressor gene RB1**, inhibits the bound transcription factor E2F and thus is significant in regulating the cell cycle, chromatin structure, proliferation and differentiation of tumour cells and cell death [30, 31]. Lost expression of the gene *RB1* plays a role in the pathogenesis of tumour development. This is due to inactivation of deletion alleles, point mutation or hypermethylation of its promoter, increased expression and/or amplification of the gene for cyclin D1, decreased expression of inhibitor p16INK4A or binding protein pRb by oncoprotein E7 HPV. In addition, the concurrent presence of the mutation in genes *RB1* and *TP53* induces the process of epithelial-mesenchymal transition (EMT). In TNBC with confirmed inactivation of the *RB1* gene, this leads to induction of increased sensitivity to radiotherapy and cytotoxic drugs, such as doxorubicin, methotrexate or inhibitor of mitochondrial translation of proteins, tigecyclin. Inhibition of expression of glucose 1 transporter (GLUT1) by tumour cells with confirmed mutation of the *RB1* gene presents a new promising therapeutic goal.

2.2 Regulators of cell proliferation, migration and angiogenesis

The loss of effective mechanisms to repair damaged DNA during the cell cycle leads to uncontrolled cell division and their tumour transformation. Adequate nutrition for the tumour cells is provided by the process of angiogenesis. To initiate the metastatic cascade, there must be an increased expression of proteases by tumour cells with subsequent degradation of the basal membrane. Cells of the tumour stroma may amplify the aggressive potential of the tumour even further and thus participate in the EMT process.

Fibroblast growth factor receptor (FGFR) consists of an extracellular domain, transmembrane domain and an intracellular domain with tyrosine kinase activity [32–34]. The binding of fibroblast growth factor and its cofactor to the extracellular portion of one of four evolutionally conserved receptors leads to the dimerization of its polypeptide chain, autophosphorylation and subsequent activation of signalling molecules, which influence cell proliferation and differentiation (MAPK, PI3K-AKT), inflammatory reaction (MAPK – kinase p38, JNK), angiogenesis (MAPK - kinase p38; PI3K-AKT - FOXO1, TSC2), apoptosis (MAPK – JNK; PI3K-AKT - FOXO1, TSC2) and cell growth, metabolism and motility (PLC γ – IP3 – DAG, PKC). The therapeutic response to the administered FGFR inhibitors (for example dovitinib, lucitanib) differs greatly. While in cases of confirmed fusion of the genes *FGFR3-TACC3* or amplification of the gene for FGFR1, an excellent therapeutic response is observed, the mutation in specific genes is associated with a significantly reduced to zero therapeutic response. In breast cancer, the aberrant activation of receptors FGFR1 and FGFR4 is associated with resistance to chemotherapy (doxorubicin, cyclophosphamide), endocrine therapy (tamoxifen, fulvestrant) and VEGF inhibitors (bevacizumab).

Epidermal growth factor receptor (EGFR) consists of a glycoprotein with an extracellular domain for ligand binding (EGF and TNF- α), a transmembrane domain and cytoplasmic domain with tyrosine kinase activity [35–37]. The EGFR/ ErbB1 receptor is significantly involved in the regulation of the cell cycle, cell migration, proliferation, differentiation and survival, by way of activating its secondary signalling pathways Ras/Raf/MEK/ERK, Ras/PI3K/AKT1/mTOR or Src/STAT3. After translocation to the nucleus, EGFR/ErbB1 regulates transcription and repair of damaged DNA. Aberrant activation and increased expression of EGFR/ ErbB1 is caused by amplification or mutation of its gene. Increased expression of EGFR was proven in 13–76% of TNBC, whereas amplification of the gene in only 2–24% of cases and is more frequent in patients with mutation in the *BRCA1* gene. An increased number of copies of the *EGFR* gene was found in 8–27% TNBC. The use of EGFR inhibitors in monotherapy or in combination with chemotherapy is being considered especially in advanced and generalised forms of TNBC. The combination of docetaxel with cetuximab seems to be effective. In patients treated with a combination of cisplatin and cetuximab, a correlation between therapeutic response and intensity of CD8+ lymphocyte infiltration (tumour infiltrating lymphocytes – TILs) has been reported. Combination therapy with PARP inhibitors or immune checkpoint inhibitors (PD-1/PD-L1) has promising therapeutic potential. The synergistic effect of anti – EGFR therapy was also noted with radiation therapy. A possible mechanism of resistance development to EGFR inhibitors is methylation of the extracellular domain of the EGFR/ErbB1 receptor by protein PRMT1 or increased expression of the Notch3 protein.

Vascular endothelial growth factor (VEGF) binds to its specific transmembrane receptors with tyrosine kinase activity (VEGFR-1 and VEGFR-2) by activating matrix metalloproteinases (MMP) and stimulates cell migration and endothelium proliferation with the creation of vascular lumen and fenestrations [38, 39]. Unregulated angiogenesis may be induced by genetic changes (mutations in tumour suppressor genes *TP53* or *VHL*, activation of oncogenes), as well as metabolic changes (hypoxia, effect of gonadal hormones, growth factors and cytokines). Increased VEGF expression is often observed in patients with advanced disease stages, resistant to therapy or with a mutation in the *BRCA1/2* genes. In TNBC, a synergic anti – angiogenic effect of the intravenously administered AAV2-VEGF-Trap and paclitaxel has been detected. Coenzyme Q0 has a similar effect, whereby its effect on signalling pathway PI3K/AKT/NFKB/MMP-9 and negative regulation of MMP-2/–9, urokinase activator of plasminogen (uPA), receptors

uPAR and VEGF lead to induction of apoptosis and inhibition of EMT. In advanced and metastasizing forms of TNBC, the benefits of combination therapy of bevacizumab and chemotherapy or mTOR inhibitors (temsirolimus, everolimus) or EGFR (erlotinib) are also being considered.

2.3 Proteins regulating apoptosis

Cell death receptors Fas and TRAIL of the tumour necrotizing factor (TNF) family are considered to be potential anti-tumour molecules. The **Fas receptor (CD95R)** is a transmembrane protein, composed of an extracellular, transmembrane and intracellular domain [40]. Binding the soluble membrane ligand of cytotoxic T-lymphocytes CD95 (CD95L, FasL) leads to the creation of complex DISC and activates the extrinsic apoptosis pathway. Soluble ligand CD95L, labelled cl-CD95L, is responsible for activating the immune response, EGFR and the oncogenic signalling pathway c-yes/Ca2+/PI3K. Increased expression of CD95R was found in almost 49% of TNBC. Decreased expression of the Fas receptor (CD95R) is a marker of poor prognosis. Expression of CD95L by tumour blood vessels and detection of serum levels of cl-CD95L predicts metastatic potential of the tumour in patients with TNBC. Excessive expression of protein Lifeguard by TNBC tumour cells inhibits the activity of CD95R receptor and thus presents a possible mechanism of resistance to systemic therapy with cisplatin. The **TRAIL receptor ligand (Apo2L)** activates the extrinsic apoptosis pathway in the mesenchymal subtype of TNBC [41]. Agonists of the TRAIL (TNF - related apoptosis -inducing ligand) receptor stimulate death receptors DR4 and/or DR5. In advanced and metastasizing forms of TNBC, molecule MEDI3039 has shown a positive therapeutic effect.

Gene TP53 with tumour suppressor function plays the role of genome guardian. Its product, **protein p53**, acts as a transcription factor following translocation to the nucleus and has a fundamental influence on the regulation of checkpoints of the cell cycle, cellular response to damaged DNA and telomeres, aerobic cell metabolism, apoptosis, inhibition of angiogenesis and oncogene activation [42]. Protein p53 consists of an N – terminal domain activating transcription, DNA binding domain, oligomerization domain and protease-sensitive domain, which enables the binding of p53 to damaged DNA. Functional protein p53 exists in the form of a tetramer, where loss of function of one subunit causes nonfunction of the entire complex. Mutations in the *TP53* gene were discovered in 60–88% of TNBC. They are considered as a negative prognostic and predictive marker in terms of disease-free survival (DFS), overall survival (OS) and therapeutic response to chemotherapy. Manipulating genes involved in the regulation of protein p53 and its isoforms (Cyclin G2, Sharp-1, PI3K/AKT/mTOR, Chk1, CDK, Hsp90, Mdm2, histone deacetylase) may lead to new therapeutic strategies for TNBC.

Anti-apoptotic protein Bcl-2 is reported to be an independent negative prognostic marker of survival in patients with TNBC [43–45]. Expression of protein Bcl-2 in TNBC positively correlates with the size of the tumour and the development of metastases to regional axillary lymph nodes. It is also associated with a lower sensitivity to neoadjuvant and adjuvant chemotherapy with anthracyclines and resistance of the tumour to radiation therapy due to activation of the *STAT3* gene. The use of Bcl-2 inhibitors may have a protective effect against resistance development to chemotherapy and immunotherapy.

2.4 Regulation of gene expression

Detection of epigenetic changes taking place in breast cancer may aid in determining disease prognosis and in predicting the response to treatment. These primarily

include changes in DNA methylation, modification of histones and altering miRNA expression [46–53]. Recently, the regulatory role of lncRNA, circRNA and siRNA has been described.

DNA methylation is among the most important modifications, ensured by the action of DNA methyltransferases, regulated by genes *DNMT1*, *DNMT3a* and especially *DNMT3b*. Also associated with the development and progression of breast cancer is hypermethylation of CpG promoters of tumour suppressor genes (*RASSF1A*, *CDKN2A*, *CDKN1B*, *CCND2a*), genes regulating repair of damaged DNA (*BRCA1*, *MLH1*, *MGMT*), cellular detoxification genes (*GSTP1*), adhesion, invasion (*TWIST1*, *CDH1*, *TIMP3*), hormone receptors (*ER*, *PR*) and apoptosis (*HOXA5*, *TMS1*).

Post-translational modification of histones includes their phosphorylation, ubiquitination, methylation and demethylation, acetylation and deacetylation. Methylation of histone H3K27 by protein EZH2 is described in aggressive and metastasizing forms of breast cancer. A therapeutic response may be reached using histone deacetylase inhibitors (vorinostat, entinostat and panobinostat) in monotherapy or in combination with cytotoxic, hormonal or targeted anti - HER2 and anti - VEGF therapy.

MiRNA represent endogenous short non-coding single strand RNA molecules with a length of 18 to 25 nucleotides. The miRNA sequence is phylogenetically conserved. They are partially or completely complementary to one or more mediator RNA (mRNA) and may also regulate other miRNAs. MiRNAs are significant regulators of gene expression and participate in the regulation of more than 50% of human genes. They are involved in angiogenesis, cell growth, proliferation, differentiation, effectiveness of mechanisms of damaged DNA repair and apoptosis. Changes in miRNA expression are therefore responsible for the development of many diseases, including dysfunctions of the immune system, tumours or resistance to pharmacological or radiation therapy. Depending on their role in the pathogenesis of tumour development, they can be divided into two types, miRNA with oncogenic or with tumour suppressor function. The positive influence on the EMT process also potentiates tumour metastasis. In the past years, miRNA has received much attention in connection with changes in its serum concentrations and its possible prognostic and predictive potential.

The miRNA biosynthesis is predominantly enabled by two major pathways - canonical and non-canonical pathway. The first pathway is initiated by the generation of the pri-miRNA transcript which is cleaved by microprocessor complex (Drosha and DGCR8) into precursor-miRNA (pre-miRNA). Pre-miRNA is transferred by the Exportin5/RanGTP to the cytoplasm and processed by the RNase III endonuclease Dicer to produce the mature miRNA duplex. The load of 5p or 3p strands of the mature miRNA duplex into the Argonaute (AGO) family of proteins to form a miRNA-induced silencing complex (miRISC). The second pathway begins by microprocessor complex - mediated cleavage of small hairpin RNA (shRNA) with following its export to the cytoplasm via Exportin5/RanGTP. Nevertheless, the further possible pathways were identified (e.g. Dicer-independent cleavage, miirtrons and 7-methylguanine capped (m7G)-pre-miRNA formation).

Long non-coding RNA (lncRNA) are, contrarily, molecules with a length of 200 and more nucleotides. Aberrantly increased lncRNA expression is able to stimulate the oncogenic signalling pathway PI3K/AKT, as well as changes in miRNA expression. They participate in the regulation of the biological behaviour of tumours and may induce a therapeutic response to administered systemic therapy. Newly described lncRNA includes DANCER (lncRNA - differentiation antagonising non-protein coding RNA), sONE, CCAT1 or GAS5. So-called **circular RNA (circRNA)** has a similar significance.

Short interfering RNA molecules of siRNA are due to their ability to reduce the expression of protein Bcl-2 and p-glycoprotein considered to be one of the possible mechanisms for developing resistance to chemotherapy in TNBC. Formation of conjugates with nanoparticles of silicon dioxide, or in combination with chemotherapy, may enhance therapeutic possibilities in the future.

2.5 Steroid androgen receptor

The androgen receptor (AR) is a nuclear steroid hormone receptor which is expressed in 70–90% of all breast cancers [54–56]. It contains a transactivation N-terminal domain, a DNA-binding domain and a C-terminal domain. The function of AR as a transcription factor is to modulate the activity of steroid-regulated genes, or to alter post-transcription processes, which leads to changes in levels of specific mRNA and proteins. Inactive form of AR is kept in the cytoplasm by a heterocomplex with heat-shock proteins and a chaperone complex (HSP-70, HSP-90). There exist two mechanisms of AR activation – genomic modality and non-genomic modality. Genomic modality is implemented by androgen binding to the C-terminal domain of AR, its conformational change, dimerization and translocation into the nucleus, leading to a promotion of a co-activator-mediated transcription of target genes. Non – genomic modality activates AR through ERK dependent (interaction with PI3K, Src proteins, Ras GTPase) or ERK independent signal transduction (mTOR phosphorylation, FOXO1 inactivation, PKA activation)

In TNBC, increased expression of AR was observed in 10–50% of cases. Although several studies concerning ER-related breast cancers confirm a positive correlation between its increased expression and disease-free survival (DFS) as well as overall survival (OS), others claim the opposite. Expression of AR in TNBC is associated with lower grade, lower proliferation activity and lower disease stage. The lack of AR expression is thus considered to be a factor associated with a higher risk of disease recurrence and development of distant tumour metastases. Taking into account the sensitivity of the tumour to systemic therapy, the use of AR antagonists in clinical practice seems more than promising.

2.6 Immune checkpoint proteins

Physiologically, healthy tissue is protected from damage by its own immunocompetent cells by inducing immune tolerance. It is mediated by cells of the immune system (especially T – lymphocytes, B - lymphocytes, macrophages, dendritic cells), which are able to effectively detect tumour antigens and activate a cellular and humoral antitumour response. A more intense antitumour immune response correlates with longer overall patient survival, period without development of metastases, period without disease relapse and symptom-free interval.

Understanding the mechanism of how tumour cells escape from immune supervision (theory of immunosurveillance) led to the identification of immune checkpoint proteins as potential aims of immunotherapy. The signalling pathway PD1/PD-L1 under normal conditions inhibits the PI3K/Akt and MAP-kinase pathway (Ras/MEK/Er) and leads to the induction of apoptosis and termination of the cell cycle. It also limits the effector function of CD8+ T-lymphocytes in favour of regulatory CD4+ T-lymphocytes. Receptor protein PD-1 is encoded by the gene *PDCD1* on chromosome 2. Its role in the immune system is played by two ligands with co-inhibitive function, protein PD-L1 (CD274) and PD-L2 (CD273). PD-L1 is expressed on the surface of T - and B - lymphocytes, dendritic cells, macrophages, mesenchymal stem cells and mastocytes; PD-L2 is only expressed on the surface of antigen-presenting cells and mastocytes.

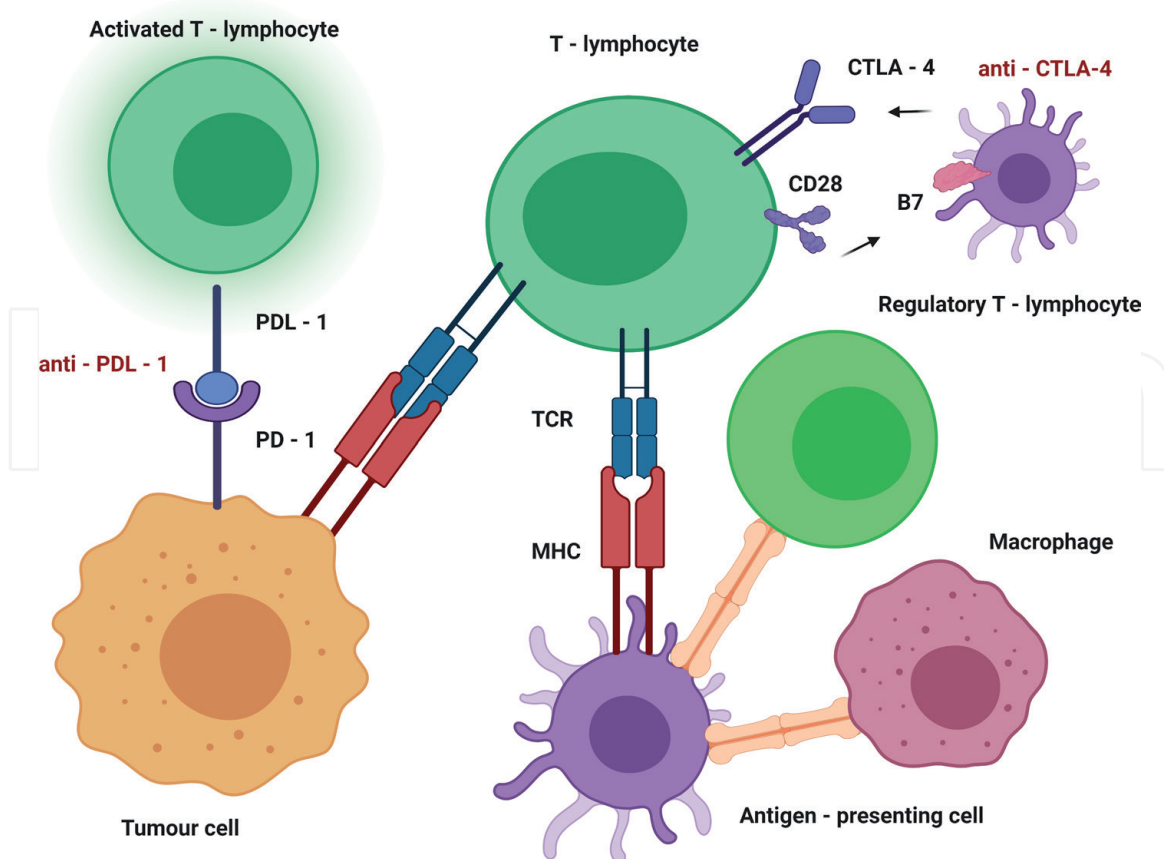


Figure 3.
 Immune response to cancer - mechanism of action of PD-1/PD-L1 (Koleckova M, www.biorender.com). The mechanism of PD-1/PD-L1 axis action is based on the controlling of the anti-tumour immune response by the self-tolerance promotion. The activity of PD-1 and its ligand (PD-L1, PD-L2) is involved in the modulation of immune system accompanied by T cell activation, proliferation, cytotoxic secretion and apoptosis. Targeting the PD1 and PDL1 immune checkpoint proteins represents the new era of therapeutic strategy.

The testing of monoclonal antibodies with anti-PD-L1 inhibitory effect and their introduction into clinical practice signified a breakthrough in the treatment of a number of tumours [57–65]. Increased expression of PD-L1 in tumour cells is generally associated with poor disease prognosis. Contrarily, its increased expression by immune system cells (TILs) prolongs overall patient survival. Increased expression was observed in 20% of TNBC cases. Expression of PD – L1 in the tumour and its metastases in the lymph nodes is very heterogeneous and changes in time. Administration of immune checkpoint inhibitors (anti – PD1 - pembrolizumab, anti – PD-L1 - atezolizumab) with cytotoxic drugs is recommended in advanced forms of TNBC. Atezolizumab in combination with nab – paclitaxel has been shown to be effective; cases with increased expression of PD-L1 reported a prolongation of progression-free survival (PFS) from 5 months to 7.5 months and overall survival (OS) from 15.5 months to 25 months. Complete pathological response (pCR) was reached in 51.9% of cases receiving atezolizumab with nab – paclitaxel and carboplatin, and in 64.8% of cases receiving pembrolizumab with nab – paclitaxel and carboplatin (**Figure 3**).

3. Conclusions and future perspectives

The issue of TNBC is still a challenge for many investigators over the world. The current scientific interest is mainly focused on the development of promising therapeutic targets. Due to poor prognosis associated with tumour aggressive

biological behaviour, high rates of metastases and unpredictable response to the primary systemic chemotherapy and radiotherapy, the detailed analysis of the mechanisms of TNBC genesis is asked. Identification of new potential targets and the development of specific targeted therapy is pivotal for improvement of the existing clinical outcomes. The knowledge of the crucial participation of immune system in carcinogenesis significantly extended the range of therapeutic options. Ongoing clinical trials testing different types of molecules may pave the way for effective pharmacological synergy and better treatment results.

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