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The significance of the pluripotency and cancer stem cell-related marker NANOG in diagnosis and treatment of ovarian carcinoma

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Summary

Ovarian cancer is among the most common gynecologic cancers and unfortunately the most common cause of death from gynecologic malignancies. Due to few early symptoms and insufficient screening programs, an early diagnosis of ovarian cancer is very difficult and new biomarkers related to early ovarian carcinogenesis are needed. In the last years a growing scientific knowledge about cancer stem cells and their markers opened a new perspective on screening and early diagnosis of ovarian cancer. The transcription factor NANOG is not only a pluripotency and cancer stem cell-related marker, but also promotes cancer stem cell-like characteristics of tumor, tumor growth, dissemination, immune evasion, and resistance to conventional therapy. The recent data showed that small stem cells resembling very small embryonic-like stem cells are present in the ovarian surface epithelium of adult human ovaries. These cells expressed several genes related to primordial germ cells, germinal lineage, and pluripotency, including NANOG, therefore their involvement in the manifestation of ovarian cancer are not excluded. As majority of cancer cells within a tumor are non tumorigenic, the therapies targeting these cells cause tumor regression, but the survived cancer stem cells regenerate the tumor, so tumor relapse or reoccur. The eradication of cancer actually requires the elimination of cancer stem cells, therefore new strategies in treatment that specifically target cancer stem cells are urgently needed. Although the therapeutic efficacy of targeting NANOG as a cancer treatment method is still in experimental phase, the gene therapy with small interfering RNA or short hairpin RNA have already shown some promising therapeutic potential. The authors can conclude that NANOG represents a promising diagnostic marker and agent for target therapy of ovarian cancer.

Key words: Ovarian cancer; Cancer stem cells; NANOG; Chemoresistance; Target therapy.

Introduction

Ovarian cancer is the most common cause of death from gynecologic malignancies, and the second most common gynecologic cancer; the incidence in Slovenia is 16/100 000 females [1]. Due to few early symptoms and insufficient screening program, an early diagnosis is very difficult [2, 3]. Consequently majority of ovarian cancer is still diagnosed at advanced stage. At the time of diagnosis 77.8% of women have an advanced disease with tumor spreading outside the pelvis [1]. Five-year survival rate for advanced tumor is less than 30% with only modest improved survival over the past 40 years [1]. Although majority of advanced-stage patients respond to standard chemotherapy, tumor relapse occurs in over 70% of patients, resulting in chemoresistant and fatal disease.

Pathogenesis of ovarian cancer

Ovarian cancer represents a heterogeneous disease composed of different types of tumors. Approximately 90% of ovarian cancers belong to the group of epithelial ovarian cancers (EOC). The majority 75% of epithelial ovarian cancers are of the serous histologic type. Less common types are mucinous (20%) and endometrioid carcinomas (2%). Clear cell, transitional cell (Brenner), squamous cell, mixed epithelial, undifferentiated, and unclassified carcinomas are each representing less than 1% of epithelial lesions [2, 3].

The etiology of EOC is still unknown. Approximately 15% of ovarian cancers are familial and 85% sporadic [4]. BRCA1 and BRCA2 mutations account for almost 90% of hereditary genetic disorders responsible for ovarian cancer. The remaining 10% of hereditary ovarian cancers include Lynch syndrome and hereditary nonpolyposis colorectal cancer (HNPCC) [5]. Slow growing tumors (low-grade micropapillary serous carcinomas, mucinous carcinomas, endometrioid carcinomas and clear cell carcinomas) are genetically stable and are characterized by mutations in a number of different genes including KRAS,

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BRAF, PTEN, and beta-catenin [6, 7]. Rapidly growing, highly aggressive neoplasms, (high-grade serous carcinomas, malignant mixed mesodermal tumors – carcinosarcomas, and undifferentiated carcinomas) have a high level of genetic instability and are characterized mainly by mutation of *TP53* [6, 7]. The cell of origin of EOC and the mechanisms of carcinogenesis have been long debated and more hypotheses have been proposed.

New theories about the cell of origin and manifestation of EOC

By tradition, all aspects of EOC are derived from the ovarian surface epithelium (mesothelium) and that subsequent metaplastic changes lead to the development of the different cell types (serous, endometrioid, clear cell, mucinous, and transitional cell) which morphologically resemble the epithelia of the fallopian tube, endometrium, endocervix, gastrointestinal tract, and urinary bladder.

Recently fimbrial-ovarian theory for development of EOC has been proposed. Direct implantation of tubal epithelium into the ovary to form an inclusion cyst, which is the site of origin of ovarian serous carcinoma, is an attractive alternative theory to that of metaplasia from the surface epithelium (mesothelium) [6]. Implantation of fallopian tube epithelium from the fimbria at the time of ovulation when the surface epithelium is disrupted can explain the derivation of low- and high-grade serous carcinomas [6]. In the case of a low-grade serous carcinoma, which accounts for approximately 10% of serous carcinomas, the process develops slowly from a serous cystadenoma, serous borderline tumor, serous non-invasive micropapillary carcinoma to low-grade serous carcinoma [6, 7]. High-grade serous carcinomas, which accounts for approximately 90% of serous carcinomas, arise from high-grade intraepithelial serous carcinomas in the fimbriated end of the fallopian tube which then spread to the ovary. This tumors are highly aggressive, rapidly growing, and disseminating, with sonographycally undetected precursor lesions [6, 7]. According to this theory, both low- and high-grade serous carcinomas are of tubal (Müllerian) origin and the ovary is involved secondarily.

Endometrioid and clear cell tumors have been associated with endometriosis. Endometriotic cysts, frequently associated with implants of endometriosis elsewhere in pelvis, are regarded as the precursor lesions for these tumors [7]. Finally, as preliminary data suggest, mucinous and transitional (Brenner) tumors arise from transitional-type epithelial nests at the tubal-mesothelial junction by a process of metaplasia [7].

In summary, theories mentioned above have something to offer in explaining the development of ovarian carcinomas but none can explain it completely. It looks like in EOC ovaries are involved in fact secondarily.

Diagnosis of ovarian cancer

For the last decades several studies have been performed in an effort to develop screening tests for ovarian cancer in order to detect tumor when it is still confined to the ovaries. The modalities that are currently used for screening are pelvic examination, transvaginal ultrasound, and serum CA 125.

Slow growing tumors, like low-grade serous carcinoma, low-grade endometrioid carcinoma, clear cell, and mucinous carcinoma achieve large sizes while still confined to the ovary. At that point they can be detected by pelvic examination and/or transvaginal ultrasound. However, they account for only 25% of ovarian cancers and approximately 10% of ovarian cancer deaths [8]. For these type of tumors the development of a biomarker used as screening test is not so urgently needed.

High-grade serous carcinomas, undifferentiated carcinomas, and malignant mixed mesodermal tumors (carcinosarcomas), on the other hand, are highly aggressive, they spread rapidly when tumor is still small, and cannot be detected by pelvic examination and/ or transvaginal ultrasound. Consequently the majority of women with this type of tumors have already an advanced disease at the time of diagnosis [1]. Unfortunately, these tumors represent 75% of all ovarian carcinomas and are responsible for 90% of ovarian cancer deaths [8]. These tumors need a new screening approach, which will detect tumors while they are still small and confined to the ovary. This can only be achieved by developing a sensitive and specific biomarker that is expressed early in ovarian carcinogenesis.

Cancer stem cells

Human tissues are able to maintain their mass and architecture despite tissue damage that occurs over lifetime through precisely regulated process of renovation. This process is physiologically regulated by a minor population of long-lived cells with unlimited expansion potential, known as stem cells (SCs). SCs are characterized by ability of self-renewal and differentiation into specialized cells.

The idea that cancer is raised from a subpopulation of tumor cells with stem cell properties was proposed around 150 years ago by pathologist Rudolf Wirchow [9]. Tumors can be considered as aberrant organs that are initiated by tumorigenic cancer cells [9]. Tumorigenic cancer cells in time through several mutations acquire the capacity for indefinite proliferation giving rise to latterly termed cancer stem cells (CSCs). Tumors are composed of heterogeneous populations of cells that differ in their degree of accumulated mutations and state of differentiation [9]. American Association for Cancer Research (AACR) workshop defined CSC as a malignant cancer cell with a stem cell phenotype [10].

The etiology of CSC is still uncertain. One hypothesis is that CSCs are the malignant counterparts of normal adult tissue SCs. Also their relation to germinal stem cells is not excluded. Due to their long life, SCs remain in a tissue for longer period of time compared to their differentiated progeny, making them more prone to acquire several mutations. Other hypothesis is that the dedifferentiation of mature cancer cells to a more pluripotent state could be a potential mechanism for the development of cancer cells with SC-like features.

Signaling pathways are in CSCs in contrast to the normal SCs deregulated, so they are unable to maintain stem cell homeostasis [11, 12]. As well as the normal SCs, CSCs are thought to reside at the top of the hierarchy and give rise to differentiated cells, which themselves have no potential for self-renewal, and therefore do not contribute significantly to tumor growth. CSCs as well as normal SCs divide asymmetrically, so by self-renewal maintain their pool on one hand and on the other hand generate an identical daughter cell that is committed to differentiation. It has been suggested that by this mechanism CSCs in tumor enable tumor self-renewal and generate different cell types within a tumor that leads to tumor heterogeneity. This unique subpopulation of cancer cells is capable of propagating the tumor as well as developing malignant behaviors like metastasis [13-17]. Like normal SCs, CSCs represents only small proportion of cellular population [18-20], so we need specific cell markers to isolate CSCs population [21-23].

Germinal stem cells

The recent data also show that small stem cells resembling very small embryonic-like stem cells (VSELs) are present in healthy adult human ovaries. These cells express several genes related to primordial germ cells, germinal lineage, and pluripotency (including NANOG) and their involvement in the manifestation of ovarian cancer is not excluded [24, 25]. These stem cells may begin to develop into the tumor/cancer at the inappropriate conditions in the body.

Cancer stem cell markers

In last two decades, the existence and the identity of CSCs have been identified, first in hematopoietic tumors and later in solid tumors including ovarian cancer [18, 20, 26-37]. The first solid tumor from which CSCs were isolated was breast cancer: the breast CSCs were characterized as a population of less than 5% of cells expressing a high level of CD44 and a low level of CD24 cell surface markers and were positive for epithelial cell surface antigen [18, 19].

Using cell surface markers expression to characterize CSCs requires knowledge of cell surface markers expressed by the putative CSCs in specific tissue. Expression of CSCs cell surface markers is usually inferred by expression of markers in normal adult SCs. Most commonly used surface markers that are thought to be SC specific are CD133 and CD44 [38]. CD133 and CD44 surface markers have been used successfully to isolate SCs in normal and tumor tissue including ovarian cancer [37-39], though it is not clear if they characterize CSCs derived from all types of tumors. The other problem is that their expression may not be restricted only to the CSCs population and may be present in early progenitor cells too. Since CSCs surface markers do not characterize tumor initiating cells exclusively, CSCs surface markers can be used as indicators, but not a reliable marker for defining a population of CSCs in solid tumors. To increase the sensitivities and specificities for the detection of CSCs, further investigations are needed [40]. Recently there have been many reports on identifying other CSC markers, including transcription factors, stemless-related signaling pathways, and microRNAs (miRNAs).

NANOG – marker related to pluripotency and cancer stem cells

NANOG is a homeodomain-containing transcription factor and along with transcription factors OCT4 and SOX2, plays a key role in the maintenance of pluripotency and self-renewal in undifferentiated embryonic stem cells (ESCs) [41-47]. The NANOG protein is encoded by the only open reading frame of the 2184-nucleotide NANOG cDNA [41]. Beside embryonic NANOG gene (eNANOG), there are ten NANOG pseudogenes and one tandem duplication [48]. Only the NANOG homeobox pseudogene 8 (NANOGP8) has a complete open reading frame to transcribe and translate a functional NANOG protein [48-50]. The protein derived from NANOGP8 is almost identical to that from eNANOG, it has only one amino acid alteration (from Gln-253 in eNANOG to His-253 in NANOGP8) [20]. At first, in case of prostate cancer, it was presumed that eNANOG is an important regulator of pluripotency while NANOGP8 plays a role in tumorigenesis [51], but recent study on colorectal cancer revealed that in tumors, the protein NANOG can be expressed from both eNANOG and NANOGP8 [20]. Human NANOG protein consists of 305 amino acids [41, 42] and contains an N-terminal domain (amino acid 1–95), a DNA –binding homeodomain (amino acid 96–155), and C-terminal (amino acid 156–305) region consisting of two transactivation domains [41, 52]. The N-terminal domain is responsible for the transcriptional activity of NANOG. This region is tightly epigenetically regulated through phosphorylation or other post-translational modifications [52, 53]. Its N- and C-terminal domains contain nuclear localization sequences [53] though its middle region holds a potent nuclear export motif [54] allowing NANOG to transport in and out of the nucleus. NANOG mRNA is present in stem cell lines and ab-

sent from differentiated cells [42]. NANOG expression is crucial to maintain ESC identity [42], suggesting that NANOG acts as a gatekeeper of pluripotency in human embryonic development.

The expression of NANOG has been regulated by OCT4/SOX2 transcription factors [55]. NANOG together with OCT4/SOX2 form a core regulatory network that coordinately determines the self-renewal and differentiation of ESCs [44]. NANOG is also one of the key transcription factors that could reprogram a human somatic cell into an ESC-like pluripotent cell, termed inducible pluripotent SCs [54, 56].

Overexpression of NANOG has been detected in germ cell tumors as well as other tumors, including ovarian cancer [33-36, 57-60]. Moreover overexpression of NANOG protein in ovarian tumors was associated with high grade tumors, advanced clinical stage [33, 35], and shorter patient survival rate [34, 35]. The NANOG protein can bind to the promoter region of cyclin D1 and regulate cell cycle and proliferation [61, 62]. NANOG positively regulates cancer cell motility and tumor metastasis capability. It was found highly expressed in ovarian cancer cell lines with metastasis-associated property and in clinical samples of metastatic foci. Knockdown of NANOG impeded cell proliferation, migration, and invasion [35]. Overexpression of NANOG in ovarian cancer is also associated with resistance to standard chemotherapy [34, 35].

Recently it was demonstrated that the NANOG-mediated activation of Akt pathway causes cancer cells to adapt to host immune system and escape from the immune-mediated clearance [63]. The failure of cancer vaccination may be due to a NANOG-dependent evolution of tumor cells toward an immune-resistant and stem-like phenotype. It was also demonstrated that even the cancer stromal cells express high levels of cytoplasmic NANOG that may promote cancer progression [64], suggesting a role of NANOG in regulating the cross talk between cancer cells and cancer associated stromal cells.

Conventional treatment of ovarian cancer

Standard treatment of advanced disease remains combination of surgical approach and chemotherapy [65].

Surgery

The purpose of surgery is to achieve both correct International Federation of Gynecology and Obstetrics (FIGO) staging [66] and therapeutic cytoreduction. Aim of the surgical procedure is optimal cytoreduction where tumor burden is reduced to where no macroscopic tumor is left [67]. Optimal cytoreductive surgery is an independent prognostic factor [68]. When optimal cytoreduction is not possible ,surgery is performed after neoadjuvant chemotherapy [69].

Primary chemotherapy

Standard adjuvant chemotherapy after cytoreductive surgery is six cycles of carboplatin and paclitaxel. Both agents induce apoptosis. Carboplatin is an alkylating agent that binds covalently to DNA. Paclitaxel binds noncovalently to microtubules, stabilizes them, and so interferes with the normal breakdown of microtubules during cell division. Empirical addition of liposomal doxorubicin, topotecan or gemcitabine to standard therapy failed to improve progression-free and overall survival time observed with paclitaxel and carboplatin alone [70]. Vascular endothelial growth factor (VEGF)-binding antiangiogenic antibody, bevacizumab, added to standard treatment, improved progression-free but not overall survival time [71, 72].

Chemotherapy for recurrent ovarian cancer

Patients initially respond to conventional chemotherapy, but more than 70% of patients with advanced ovarian cancer will experience disease recurrence. Despite attempts with multiple lines of chemotherapy, the response rate of recurrent disease is still disappointing. When disease reoccurs more than six months after standard chemotherapy retreatment with carboplatin and paclitaxel responds in a 20–50% cases. Disease that reoccurs in less than six months is considered platinum resistant. In this case response rates are even lower, ranging from 10–30%.

Despite aggressive surgical approach and several cycles of chemotherapy with carboplatin and paclitaxel at maximum tolerated doses, sometimes in combination with other drugs, five-year survival rate in advanced disease is still less than 30% [1]. Although the standard combination of surgery and chemotherapy can at first effectively reduce tumor mass, most patients, eventually, acquire chemo-resistance [73, 74].

Role of transcription factor NANOG in resistance to conventional treatment of ovarian cancer

It was found that transcription factor NANOG plays a role in conventional therapy of ovarian cancer. Chemoresistance can be caused by an altered uptake or efflux of drug in the target cell. Platinum compounds enter the cell primarily by passive diffusion and leaves by active efflux. Several mechanisms have been described that might facilitate the active efflux of anticancer agents. Some of most frequently studied drug transporters associated with chemoresistance in CSCs are multifunctional efflux transporters from the *ABC* gene family [75]. NANOG-STAT3-ABCB1 (ATP-binding cassette sub-family B member) signaling pathway studied in breast and ovarian cancer was reported to mediate the resistance against several chemotherapeutic drugs [76]. They showed that NANOG forms a complex with the STAT3 transcription factor in the nucleus leading to *STAT3*-specific transcriptional activation and *ABCB1* gene expression [76]. These data revealed a direct regula-

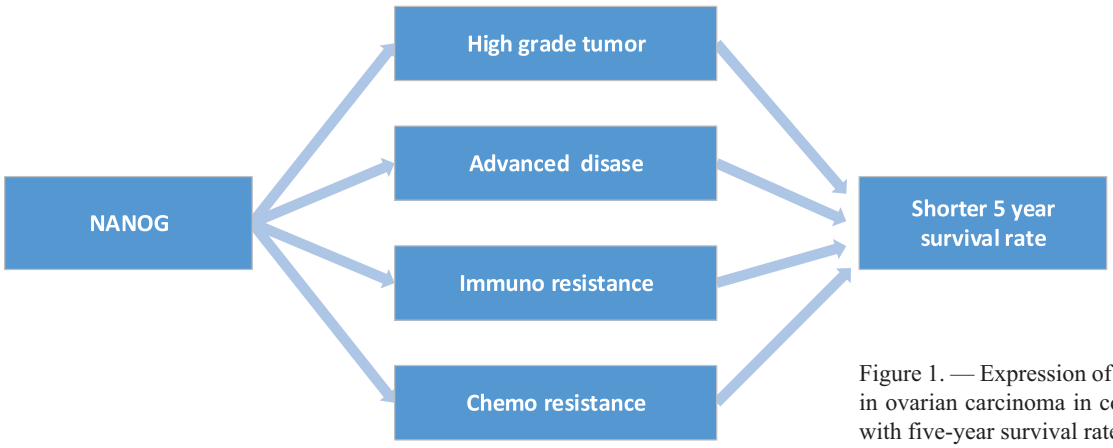

Figure 1. — Expression of NANOG in ovarian carcinoma in correlation with five-year survival rate.

tory link between NANOG and the drug-resistance mechanism in cancer cells.

The tumor suppressor gene p53 plays a critical role in cell proliferation and apoptosis. p53 also plays a role in regulation of apoptosis and chemosensitivity in human ovarian cancer cells [77-79]. Loss of p53 function relate to multidrug resistance in different types of tumors, including EOC [80]. NANOG may regulate p53-related signaling and negatively affect the pro-apoptosis mechanisms in cancer cells [62]. According to the knowledge that NANOG is involved in p53-dependent pro-apoptosis pathway and ABC genes expression, it is obvious why CSCs can escape apoptosis and are resistant to chemotherapy.

Cancer vaccination was once thought to be a promising method for treating cancer. Unfortunately the promising outcomes of immune related cancer therapy are in clinical trials so far still lacking. Cancer cells are able to adapt to the host immune system and avoid the killing and apoptosis mediated by the $CD8^+$ cytotoxic T lymphocytes [63, 81]. It has been found that application of cancer vaccination caused overexpression of NANOG [81]. Overexpression of NANOG drove tumor cells toward an immune-resistant and stem-like phenotype [81]. These findings showed an important role of NANOG in regulating the relationship between cancer cells and host immune cells, where NANOG enable cancer cells to avoid the attack from immune system. On the other hand, application of small interfering RNA (siRNA) against NANOG in these tumor cells reversed the immune-resistant phenotype and consequently reduced tumor growth [81]. This finding gives rise to a potentially new therapeutic approach, targeting NANOG for achieving immune-based therapy (Figure 1).

New therapeutic approach of ovarian cancer- targeting CSC and the role of NANOG

So far neither standard nor new therapeutic agents that are used in clinical practice have significantly improved survival rate or reduce adverse side effects. New strategies in treating ovarian cancer are needed. As already mentioned, the majority of cancer cells forming tumor are non-tumorigenic, therapies targeting these cells at first cause tumor regression, but surviving CSCs regenerate the tumor and tumor relapse or recurrence. For that reason, cancer can be successfully treated only by targeting CSCs. New strategies that will specifically target CSCs are urgently needed.

Target therapy with antibody against CSC antigens acts through inhibition of specific signaling pathways in which tumor marker is involved. Antibodies can be also conjugated with a bioactive drug and so enable selective targeting of chemotherapeutic agents. CD44 is a surface adhesion molecule that binds with hyaluronic acid. Hyaluronic acid in combination with paclitaxel enhances selective entry of cytotoxic drugs into human EOC cells expressing CD44. This has been studied in intraperitoneal treatment of ovarian carcinoma [82]. Short hairpin RNA (shRNA) was successfully applied to reduce CD24 expression. The knockdown of CD24 in ovarian cell line SKOV3 decreased cell viability by activation of apoptosis in vitro and suppressed tumor growth in mice bearing ovarian cancer in vivo [83]. CD24 inhibition can be considered as an effective approach for cancer therapy.

Target delivery of drug agent can be also provided by therapeutic nanoparticles (TNPs). TNPs are therapeutic elements combined with a drug-delivery molecule [84]. TNPs have the ability to carry a drug without affecting the carrier molecule and the ability to maneuver itself within tumor tissue. The drug-delivery molecule can also influence the speed of drug release. TNPs use the increase permeability and retention effect of immature tumor vasculature to localize tumor tissue. TNPs can be endocytosed by target cells and so escape resistance mechanisms like cell surface protein pumps. Targeted release and use of surface modifications allow anticancer drug to direct interfere with CSCs. In an in vivo mouse model of ovarian cancer, nanoliposomal siRNA was used for ALDH1A1

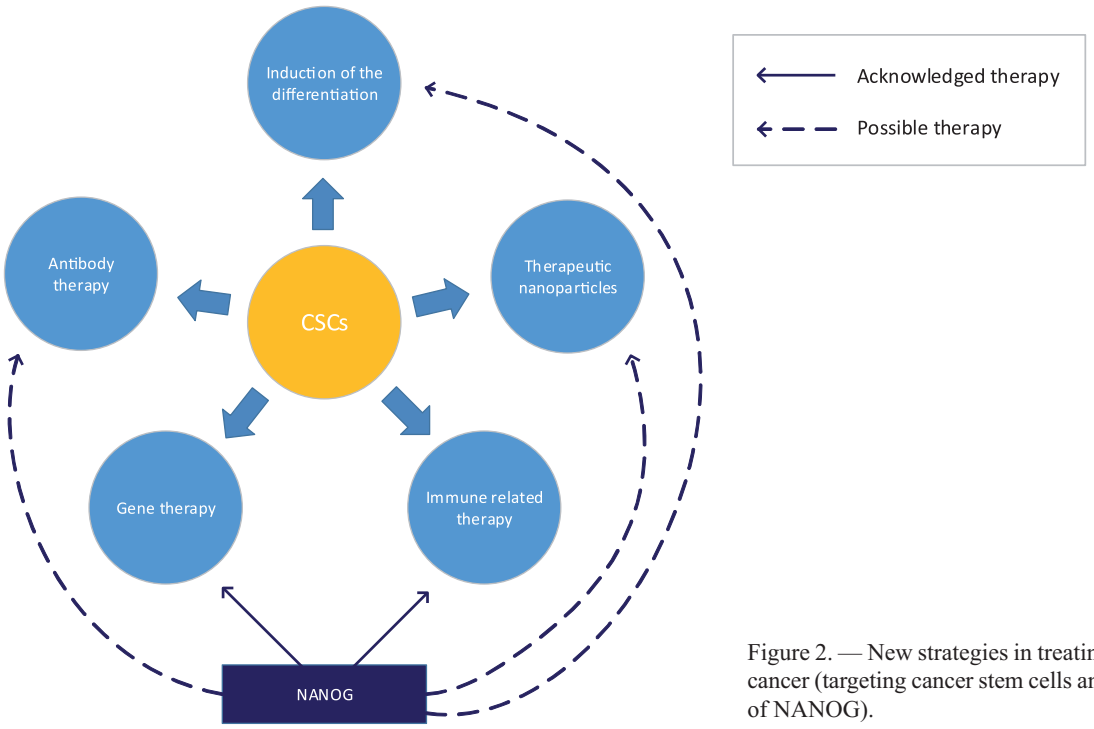

Figure 2. — New strategies in treating ovarian cancer (targeting cancer stem cells and the role of NANOG).

silencing in CSCs to sensitize both taxane- and platinum-resistant cell lines to chemotherapy. It significantly reduced tumor growth compared to chemotherapy alone [85].

Recently a new strategy of targeting CSCs has emerged. Instead of removing CSCs in tumors induction of the differentiation can be done. CSC differentiation can be stimulated by retinoic acid. Retinoic acid and its analogs are used since they are modulators of differentiation and proliferation of epithelial cells. Recently carboplatin combined with three novel retinoid compounds were used to significantly reduce growth of ovarian CSC [86]. Specific unsaturated fatty acids (palmitoleic, oleic, and linoleic acids) can also trigger adipocyte-like differentiation in cancer cells, including ovarian cancer cells [87]. Recently it was reported that p53 can activate microRNA (miRNA) [88], a small noncoding RNA that could regulate gene expression at post-transcriptional level. MiRNAs are involved in self-renewal and differentiation of embryonic SCs and CSCs [88] and might represent a therapeutic target for cancer treatment [89].

Gene therapy is another promising therapeutic method for cancer. Application of exogenous DNA or RNA sequence into host cells enables to enhance or suppress the expression of a selected gene and block the deregulated signaling pathway. As NANOG is involved in several well-known oncogenic pathways, it can be chosen as therapeutic target. NANOG suppression inhibits CSC functions, reduces tumor growth and metastasis, prevents resistance to chemotherapeutic drugs, and enhances the immune surveillance of the host. NANOG should not be expressed in differentiated somatic cells, so adverse side effects of NANOG-targeting should be limited. There are several reports on using RNA mediated NANOG-knockdown in different cancer models [51, 61, 90]. Using NANOG siRNA in cancer cells showed a synergistic therapeutic effect with chemotherapeutic drugs such as cisplatin [91]. Inhibition of NANOG decreases chemoresistance of cancers since NANOG has been linked to chemoresistance through ABCB1 and p53 mechanisms [76, 92, 93]. Furthermore, delivery of NANOG siRNA into tumor-bearing mice increased tumor exposure to immune system and suppressed tumor growth [81]. Combining NANOG-knockdown with current therapeutic methods, like cisplatin and cancer vaccination, could improve the therapeutic outcome (Figure 2).

Conclusion

NANOG regulates the cell outcome in both ESC and CSC. NANOG is implicated in several signaling pathways, both pro-oncogenic and tumor-suppressive. Moreover NANOG mediates the communication between cancer cells and surrounding stroma. NANOG also regulates the relationship between cancer cells and host immune cells. That is why NANOG could represent ideal target for cancer treatment. Therapeutic efficiency of NANOG knockdown in controlling tumor growth, metastasis, drug-resistance, and immune evasion in animal models looks promising, but introduction of new treating strategy in clinical practice might bring up

many challenges. CSCs share epitopes with normal SCs so drugs targeting CSCs may harm normal SCs and increase the risk of toxicity and adverse side effects. An ideal therapeutic agent should selectively target CSCs above normal SCs. CSC target therapies target only a small fraction of cells within the tumor, not the whole bulk of the tumor, so response to treatment may require some time to become visible. Therefore it would be beneficial to combine target therapy with cytotoxic chemotherapy to reduce also the proliferating bulk of tumor. The treatment success should be estimated by both shrinking of the tumor bulk and eradication of CSCs population for which new monitoring technics should be designed that could be used in clinical practice.

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