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Neuroendocrine differentiation in prostate carcinoma: focusing on its pathophysiologic mechanisms and pathological features

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SUMMARY: **Neuroendocrine differentiation in prostate carcinoma: focusing on its pathophysiologic mechanisms and pathological features.**

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Prostate carcinoma, even at advanced stages, responds in most patients to androgen deprivation therapies, that are able to exploit the androgen-sensitivity of prostate cancer cells. However, more than half of such tumors, within one to three years, escape these treatments, thus progressing to the hormone-refractory condition. Intriguing links between the development of hormone-insensitivity and neuroendocrine (NE) differentiation in prostate carcinoma have been hypothesized. While, some time ago, NE cells have been considered as derived from progenitor neural crest cells, currently are thought to arise, as well as both basal and secretory cells of prostate gland, from common pluripotent stem cells. NE cell are nonproliferative, terminally differentiated, PSA/acid phosphatase and androgen receptor (AR)-negative cells, moreover exhibiting an antiapoptotic phenotype due to survivin expression. They secrete a wide range of peptide hormones and biogenic amine serotonin and express neuronal markers such as chromogranins A, B, C (CgA, B, C) and neuron specific enolase (NSE) together with synaptophysin. The propensity of prostate cancer cells to undergo a transdifferentiation pathway towards NE phenotype is due to several microenvironmental conditions such as androgen depletion (induced by LH-RH analogs or antagonists, antiandrogens, 5-α-reductase inhibitors), ionizing-radiation therapy, adrenergic factors, increase in interleukin-6 signaling cascade. NE differentiation in prostate malignancy arises in three different forms: carcinoid, oat cell carcinoma, focally NE-differentiated conventional tumor. Selective expression of stem cell-associated markers, such as CD44/Oct4A gene, in NE cancerous cells explain their therapy escape together with tumor recurrence and metastasis. Malignant NE cells, although unable to proliferate, increase the proliferation of the neighboring nonneuroendocrine cancer cells, by providing them with hormone peptide-mediated growth paracrine stimuli. Aberrantly activated glutamic acid decarboxylase-independent pathway for production of GABA (γ-amino-butyric acid) appears to be a constant feature of invasive NE tumors. Serum levels of CgA reflect NE differentiation in prostate carcinoma more suitably than those of NSE. Intriguingly, intermittent androgen deprivation therapy, by preventing NE differentiation, significantly reduces the risk of a rise in serum CgA levels meanwhile delaying the time of cancer progression due

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RIASSUNTO: **Differenziazione neuroendocrina nel carcinoma della prostata: puntualizzazione su meccanismi patogenetici e connotazioni patologiche.**

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Il carcinoma della prostata, pur negli stadi avanzati, è responsivo, nella maggior parte dei malati, alle terapie di deprivazione androgenica basate sulla androgeno-dipendenza delle cellule tumorali prostatiche. Tuttavia, più della metà di tali tumori, nell'intervallo da uno a tre anni, elude l'efficacia di queste terapie, progredendo, pertanto, alla condizione di ormono-refrattarietà. Sono state ipotizzate interessanti correlazioni tra lo sviluppo dell'ormono-insensibilità e la comparsa di differenziazione neuroendocrina (NE) nel carcinoma prostatico. Mentre fino a qualche anno fa, le cellule NE erano considerate come elementi di derivazione dalle cellule progenitrici della cresta neurale, attualmente sono ritenute trarre origine, alla stregua delle cellule basali e secretorie ghiandolari prostatiche, da comuni cellule staminali pluripotenti. Le cellule NE sono caratterizzate dall'essere non proliferative ed in fase di differenziazione terminale, prive di recettori per gli androgeni (AR-negative), non produttrici di PSA e fosfatasi acida, presentando, inoltre, un fenotipo antiapoptotico dovuto alla espressione di survivina. Tali cellule secernono un ampio spettro di ormoni peptidici e la serotonina, esprimendo, nel contempo, marcatori neuronali quali le cromogranine A, B, C (CgA, B, C) e la enolasi neurono-specifica (NSE) assieme alla sinaptofisina. La tendenza delle cellule neoplastiche prostatiche a transdifferenziarsi nel fenotipo NE è riferibile a diverse condizioni microambientali quali la deplezione di androgeni (indotta da agonisti o antagonisti LH-RH, antiandrogeni, inibitori della 5-α-reduttasi), trattamenti radianti, fattori adrenergici, accentuazione della cascata di segnali dipendenti dalla interleuchina-6. La differenziazione NE nella patologia tumorale prostatica si presenta in tre forme differenti: carcinoide, oat cell carcinoma, tumore convenzionale con foci di differenziazione NE. L'espressione selettiva di marcatori proprî delle cellule staminali, come il gene CD44/Oct4A, nelle cellule neoplastiche NE è atta a spiegare l'insensibilità alle terapie adottate nonché le recidive e la metastatizzazione. Le cellule tumorali NE, quantunque incapaci di proliferare, incrementano la proliferazione delle circostanti cellule maligne non-NE, stimolandone, con modalità paracrine, la crescita mediante gli ormoni peptidici. Costante caratteristica dei tumori NE invasivi sembra essere l'aberrante attivazione del percorso metabolico, decarbossilasi-indipendente, da acido glutammico a GABA (acido γ-amino-butirrico). I livelli sierici di CgA riflettono la differenziazione NE nel carcinoma prostatico in modo più attendibile rispetto a quelli della NSE. Desta interesse la constatazione che la terapia ablativa degli androgeni, di tipo intermittente, prevenendo la differenziazione NE e ritardando, nel contempo, la progressione del tumore verso l'ormono-refrattarietà, riduce la possibilità d'incremento sierico della CgA. Sebbene significative acquisizioni conoscitive sulla natura della

to hormone-independence. Although valuable insights into the nature of NE differentiation in prostate carcinoma have been achieved in the last decades, additional understanding is needed about its pathogenetic mechanisms in order to devise novel therapy strategies to target them.

differenziazione NE nel carcinoma prostatico siano state raggiunte nelle ultime decadi, è necessario pervenire ad una più ampia comprensione dei suoi meccanismi patogenetici al fine di individuare strategie terapeutiche innovative volte ad interferirvi in modo mirato.

KEY WORDS: Prostate carcinoma - Neuroendocrine tumor - Radiation therapy - Nevirapine - Androgen ablation.
Carcinoma della prostata - Tumore neuroendrocrino - Radioterapia - Nevirapina - Ablazione di androgeni.

In an oversimplified clinical framework of prostate malignancy, the disease evolves through a linear sequence of conditions that include primary localized tumor, recurrence after primary treatment, cancer progression with hormone-sensitive state followed by that refractory (1). Indeed prostate carcinoma, even at advanced stages, responds, with a response rate more that 80% of cases, to androgen ablation therapies, involving LH-RH agonists or antagonists and anti-androgens, which are able to exploit the androgen-sensitivity of prostate cancer cells by either lowering serum androgen levels or blocking androgen receptor (AR) activity, hence inducing a massive apoptotic cancer cell death (1-8). Nevertheless, more than half of such tumors, within 15 to 36 months, escape these treatments, thus progressing to a hormone-refractory condition meanwhile the prostate cancer cell population tending to be enriched with neuroendocrine (NE) cells (4, 9-11).

Intriguing links between NE differentiation and development of hormone-insensitivity, with rising tumor progression, in prostate malignancy, have been hypothesized.

Neuroendocrine cells: morphologic features and functional behaviour

Besides basal and secretory cells, NE cells are the third epithelial cell type of the prostate gland, specifically sharing morhological and functional characteristics with neurons present in normal prostatic tissue [2, 8]. While, some time ago, they have been considered as derived from progenitor neural crest cells, currently are thought to arise, as well as both basal and secretory cells, from common pluripotent stem cells, representing, in normal adult prostate gland, a minor epithelial cell population (less than 1%), with greater density in the periurethral ducts than in peripheral zone of the gland (7, 8, 11, 12). Morphologically, there are two NE cell types, a) *open flask-shaped cells* with apical extensions towards the glandular lumen, and b), *closed cells* without luminal extensions. Both types show dendrite-like cellular processes that may evoke a connection network with adjacent epithelial cells (6-8, 12). Moreover, NE cells may interact, in a paracrine fashion, with prostate stromal tissue. Dense core granules are specific features of NE cells, acting as storage of endogenous secretory products.

NE cells are postmitotic, nonproliferative, terminally and highly differentiated, PSA/ acid phosphatase and AR-negative cells, exhibiting an anti-apoptotic phenotype due to survivin expression while Bcl-2 negative (6, 13, 14). Functionally, prostate NE cells display *hybrid epithelial-neuro-endocrine* features, with dual properties of endocrine cells and neurons, by secreting a wide range of eutopic-orthotopic *peptides*, including calcitonin family peptides (calcitonin, calcitonin-gene-related peptide, katacalcin), neurotensin, bombesin, thyroid stimulating-like peptide, parathyroid hormone-related peptide, somatostatin, biogenic amine serotonin (5-hydroxytryptamine, 5-HT) and by expressing a number of *neuronal markers* such as neuron-specific enolase (NSE), synaptophysin and chromogranins (A, B and C-secretogranin). These secretory products can act by endocrine, paracrine, autocrine mechanisms, besides the open cell-related lumencrine way. It has been hypothesized that NE cells might be involved in the regulation of growth and differentiation of the developing prostate tissue and in modulating secretory activity in mature gland where some hormone peptides (e.g., calcitonin gene-related peptide, bombesin) and biogenic amine serotonin have growth factor-mitogen activity whereas others perform neurosecretory inhibitory functions (e.g., somatostatin) (12). Particularly, somatostatin is able to block cell secretion and to inhibit cell proliferation by inducing cell cycle arrest and apoptosis; furthermore, it opposes the secretion of pituitary growth hormone (GH) with following reduction in IGF-1 (insulin growth factor-1) release from both the liver and prostatic tissue (6, 7, 11-14).

Pathophysiology of neuroendocrine differentiation in prostate carcinoma

The propensity of prostate cancer cells to undergo a transdifferentiation pathway towards NE phenotype

TABLE 1 - PROSTATE CANCER NEUROENDOCRINE DIFFERENTIATION: SOME ETIOPATHOGENETIC CONDITIONS.

- Androgen depletion: surgical or chemical androgen deprivation (LH-RH agonists or antagonists, antiandrogens, 5-α-reductase inhibitors)
- Ionizing radiation therapy
- Long-term anticancer chemotherapy (docetaxel)
- Adrenergic agents (epinephrine, isoproterenol)
- Conditions activating IL-6 signaling cascade

has been highlighted and has been related to several cell growth/microenvironment conditions (Table 1):

— *androgen depletion*: androgen -deprivated medium induces NE transdifferentiation of androgen-sensitive LNCaP cells through a considerable increase in cell cAMP concentrations (8, 15, 16). Intriguingly, long-term culture of LNCaP cells in *androgen-free serum* or in medium enriched with either cAMP or NS-398 can induce *proto-cadherin-PC gene* over-expression that coincides with their NE transdifferentiation together with acquisition of apoptosis resistance (8, 17). Even more, short interfering RNA_S directed against AR to induce AR knockdown are able to activate NE transdifferentiation process in androgen sensitive LNCaP cell lines by increasing their levels of NSE, cytoskeletal β-tubulin III and GAFP (glial acidic fibrillary protein) (5, 18).

The expression of *adrenomedullin* (AM) — a 52-aminoacid peptide, calcitonin gene-related peptide homologue — markedly increases in LNCaP cells cultured in androgen-depleted medium, meanwhile inducing them to assume the NE phenotype (19, 20). Furthemore, *CD10-neutral endopeptidase 24.11* — cell surface enzyme expressed by prostate epithelial cells and physiologically activated by androgens to cleave neuropeptide products of NE cells — is downregulated in androgen withdrawal state, it resulting in an increase in prostate levels of neuropeptides, such as bombesin and endothelin-1, that are able to sustain prostate cancer growth and invasiveness (14, 21).

Also a chronically maintened androgen-deprivation condition (supplementation by dual 5-α-reductase inhibitor *dutasteride*) could result in a rise of androgen-indipendent LNCaP cell clones, expressing a NE phenotype (8, 22). *Finasteride*, that reduces intracellular dihydrotestosterone levels by inhibiting 5-α-reductase type-2, has been recognized as a suitable chemopreventive drug of prostate carcinoma, decreasing its prevalence by about 25% (PCPT, *prostate cancer prevention trial*), otherwise together with more than a twofold raise in high grade invasive prostate carcinomas in finasteride-treated groups when compared to controls; however, recent investigations seem point out that finasteride may increase the rate of aggressive tumors in CgA-positive (elevated CgA serum values) subjects only if its continuous treatment is applied but not in intermittent therapy (six months therapy and as many resting period) (23, 24);

— *ionizing radiation therapy*, resulting in an increase of nuclear content of phospho-CREB (cyclic AMP-response element binding protein) and in a cytoplasmic accumulation of ATF 2 (activating transcription factor 2), both conditions that can induce NE-differentiation, thus representing a novel mechanism by which prostate malignant cells survive the radiation therapy and may support cancer recurrence (25);

— *long-term anticancer chemotherapy* (docetaxel), stress-induced by anticancer drugs playing a role in NE differentiation similarly to castration therapy (3, 14, 52);

— *prolonged treatment with adrenergic agents*: indeed, epinephrine and isoproterenol can induce NE differentiation of LNCaP cell lines by increasing intra-cell cAMP levels (16);

— *exposure to factors that are able to promote IL-6 (interleukin-6) signaling cascade*. In LNCaP cell line, the treatment with IL-6 induces NE-like differentiation whereas addition of androgens is able to block IL-6-mediated PI3K (phosphatidylinositol-3-kinase) signaling pathway and NE differentiation. As well as PI3K-mediated pathway, IL-6 can promote NE differentiation by activating other mechanisms such STAT-3 (signal transducer and activator of transcription) and $MAPK_S$ (mitogen activated protein kinases)-dependent signaling transductions (6, 26, 27).

The acquisition of NE features occurs gradually under the influences of the *microenvironmental conditions*. Indeed, all aforesaid pathogenetic conditions (androgen depletion, adrenergic agents, ionizing radiation, factors activating IL-6 signaling pathway) are able to promote, through an increase in cell cAMP levels, the expression of several *neuronal transcription factors* (e.g., some members of Forkhead box-a, Foxa, that correlate with synaptophysin expression; Neuro Dl, a neuronal differentiation factor; Brn-3a, a upregulating factor of voltage-gated Na^+ channel expression in prostate cancer cells) that lead a progressive transdifferentiation of prostate cancer cells towards the NE-like phenotype (8, 28).

Pathological features

NE differentiation is more frequent in prostate carcinoma than in other genito-urinary malignancies and it arises in three different forms: carcinoid or carcinoid-like tumor, small cell (oat cell) carcinoma, focally NE-differentiated conventional prostate carcinoma. Particularly, prostate carcinoid is poorly differentiated neoplasia which displays a wide NE differentiation; prostate small cell carcinoma is an undifferentiated, extremely aggressive tumor with rapid progression and metastasis similarly to bronchogenic oat cell tumor; focal NE differentiation is present, as clusters of malignant NE cells, in virtually all cases of conventional prostate carcinoma (29, 30).

Malignant NE cells of prostate carcinoma are phenotypically similar to normal prostate NE cells — postmitotic, terminally differentiated, apoptosis inhibiting (survivin), AR-negative and therefore surviving to androgen deprivation, PSA-nonsecretive, chromogranin/NSE/synaptophysin-positive cells — only differing from them because of sharing tumor *morphological characteristics* with neighboring non-neuroendocrine cancerous cells (31, 32).

Selective expression of *stem cell-associated marker* CD44 in NE cancerous cells may support the significance of their therapy escape and of the tumor recurrence. Both Oct4A (splice variant of Oct3/4 gene) and hASH1 (human achaete-scute homolog l), that is a pivotal member of Notch pathway, are markedly co-expressed with CgA in NE tumor cell populations (Table 2) (33, 35).

Malignant NE cells, although unable to proliferate, enhance the Ki-67 (proliferation index) positivity of surrounding non-NE cancer cells by providing them with paracrine growth stimuli such as bombesin, calcitonin gene- and parathormone-related peptides, neurotensin, serotonin, so that, in most androgen-independent prostate carcinomas, non-NE cancer cells, that express the anti-apoptotic Bcl-2 protooncogene, are often localized in close proximity to NE neoplastic cells. Also interleukin-8 (IL-8), which is produced by NE tumor cells, is able to promote, by a paracrine mechanism, the proliferation of surrounding non-NE cancer cells (8, 12, 16, 36).

Moreover, neuropeptides released by NE cancerous cells, besides facilitating the development of androgen independence, are able to paradoxically reactivate AR_s although in drug-induced androgen ablation state (5, 37). In an elegant animal model, it has been shown that prostate cancer cells, under the influence of NE cell products, can escape androgen deprivation therapy and, what's more, that the implantation of mouse NE-prostate carcinoma in the flank of castrated nude mice promotes the growth of human prostate tumor cell line implanted in the opposite flank (38).

Otherwise, an aberrantly activated glutamic acid decarboxylase-independent pathway for production of GABA (γ-amino-butyric acid) and an abnormal dopa-decarboxylase membrane-associated amine oxidase-dependent process for production of imidazole-4-acetate, appear to be a constant feature of strongly invasive NE tumors, that may be identified, as meaning a poor prognosis, by molecular imaging modalities such as MR-spectroscopy and PET (8, 39).

Immunohistochemical studies show a NE tumor cell immunoreactivity for both neuroendocrine markers (CgA, NSE, synaptophysin) and neuropeptide hormones together with biogenic amine serotonin (32). Prostate cancer tissue CgA is associated with a more quick PSA progression time in patients under androgen-deprivation therapy (40).

Serum levels of CgA reflect NE differentiation more suitably than NSE, particularly using them during the follow-up of advanced prostate carcinoma when PSA lacks in value, although in poorly differentiated prostate tumors CgB might be the major component of cancerous NE cells (6, 12, 31, 40-46). Intriguingly, it

TABLE 2 - MALIGNANT NEUROENDOCRINE CELLS: MORPHOLOGIC AND FUNCTIONAL FEATURES.

• Morphology	— phenotypically similar to normal prostate NE cells (nonmitotic, terminally differentiated, AR-negative and therefore androgen-insensitive, PSA-nonsecretive, chromogranin A, B, C/synaptophysin/NSE positive), only sharing tumor morphologic characteristics with neighboring nonneuroendocrine cancer cells; — selective expression of stem-cell-associated markers such as Oct4A gene.
• Fuction	— although unable to proliferate, they enhance the proliferation of surrounding nonneuroendocrine malignant cells by peptide hormone-related growth paracrine stimuli; — exert an antiapoptotic influence, by the survivin, on the neighboring nonneuroendocrine tumor cells; — neuropeptides released by NE cancerous cells, besides facilitating the development of androgen independence, are able to aberrantly re-activate androgen receptors of prostate tumor although in androgen-deprivated state; — interleukins (particularly IL-8), produced by NE cancerous cells, increase the invasiveness of prostate carcinoma.

has been shown that *intermittent androgen-ablation* therapy significantly reduces the risk of a rise in serum CgA levels, by preventing NE differentiation of prostate cancer cells and delaying the time to cancer progression due to castration therapy resistance (44).

Hypersecretion of either aforesaid eutopic or ectopic (α- and β-chorionic gonadotropins, endorphins, enkephalins, ACTH, ecc.) peptide hormones, especially in patients with small cell carcinoma or primary carcinoid of the prostate, can sometimes induce *paraneoplastic syndromes*, such as, most frequently, those due to inappropriate secretion of parathyroid hormone-related peptide, ACTH and andidiuretic hormone (47). Unlike most NE peptide products, the *somatostatin* has anti-secretive and anti-cell growth effects, so that some somatostatin analogs (octreotide, lanreotide, vapreotide, SOM230), by directly targeting specific somatostatin receptors, may be used as inhibitors of cancer growth (3, 12, 48-51). Furthermore, the significant expression of somatostatin type-2 receptors in NE-differentiated prostate tumor, allows the receptor scintigraphy by means of ^{111}In-pentatreotide, a somatostatin analogue, of the primary prostate cancer and its metastases, more specifically than ^{18}F-fluoro-2-deoxyglucose PET (49). Even, peptide receptor radionuclide therapy — biotherapy simultaneously with selective irradiation — of either carcinoid or small cell prostate tumors and their metastases, by using somatostatin analogues ^{90}Y-DOTATOC (DOTA-Tyr-octreotide) and, more recently, ^{177}Lu-DOTATATE (DOTA-Tyr-octreotate), could be carried out as well as it occurs in the therapy of gastro-entero-pancreatic NE-tumors (50).

Emerging remarks and forecast for major research advances

In the last decades, NE differentiation in prostate carcinoma has received more and more attention with reference to its dramatic implications in prostate cancer progression and metastasis (2, 4, 6, 8, 11, 12, 30). Therefore pathological features of NE prostate tumor phenotype have been recognized as a crucial objective for basal research by US-National Cancer Institute (6, 7 , 14).

Accumulate knowledge on this subject suggests that the *prostate cancer microenvironment* is strongly dynamic, also undergoing influences of exogenous conditions (e.g., androgen ablation, radiation-therapy) that can exert a selective pressure on the neoplastic cells. Indeed, some etiopathogenetic factors of NE differentiation of prostate cancer cells have been highlighted — including androgen deprivation, ionizing radiations, prolonged administration of adrenergic agents and stress conditions, long-term anticancer chemotherapy, chronic exposure to IL-6 —, anyway *NE phenotype expression* representing an early marker associated with the development of hormone independence (15-28, 52).

NE cancer cell products, as *hormone peptides and biogenic amine serotonin*, increase the proliferation index of neighboring non-neuroendocrine cancer cells meanwhile playing an antiapoptotic role by production of survivin (13, 14, 36, 42, 54). Furthermore, NE differentiation is implicated in chemoresistance induced by EGF (Epidermal Growth Factor) in prostate malignant cells (53).

CgA appears to be a more suitable NE marker than NSE, by detecting NE phenotype either at prostate tissue level or in plasma, and has clinical usefulness in predicting the extent of NE differentiation in prostate carcinoma (29, 30, 44, 45, 54, 55). Interestingly, the intermittent androgen ablation therapy, by preventing for a while the prostate cancer NE differentiation, delays the increase in CgA serum levels (44).

A potential role of general endocrine immunohistochemical markers, in both well- and poorly-differentiated neuroendocrine tumors of different sites, has been recently suggested, even though with certain reservation, for histidine decarboxylase (HDC) and vesicular monoamine transporter 2 (vMAT2), that are involved in the byosynthesis and storage of histamine (56).

Because the NE differentiation of tumors is still relatively known pathological entity, the discrimination of the true malignant NE pattern from that of an undifferentiated tumor requires a proper qualification in identifying NE-related biochemical alterations, moreover resorting to molecular techniques (57, 59). In this way, recent observations show that increase in Wnt (Wingless gene, *Drosophila* + Int-1, *murine* protooncongene) -11 levels in prostate carcinoma facilitate the NE differentiation with tumor recurrence, such events proving to be prevented, on the contrary, by silencing Wnt-11 expression in androgen-deprivated LNCaP cells (60).

Although valuable insights into the nature of NE differentiation in prostate malignancy have been achieved by recent studies, additional understanding is needed about it to provide novel treatment strategies directed to target NE differentiation-related mechanisms in patients with advanced hormone-refractory prostate tumor (15-28, 52, 54, 61-65). Intriguingly, just on this subject, it has been recently shown that exposure of androgen-independent highly undifferentiated prostate cancer cells to *nevirapine,* a reverse transcriptase inhibitor, is able to induce their conversion into a more differentiated phenotype with AR signaling restoration and following reappearance of hormone-sensitivity condition, what could, in turn, interfere with NE differentiation in prostate carcinoma (66).

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