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AR AND CgA *in situ* DETECTION AS PROGNOSTIC CLINICAL MARKERS IN PROSTATE ADENOCARCINOMA

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Abstract*- *Prostate cancer is the most frequently diagnosed malignancy in men. An early diagnosis of neuroendocrine differentiation in prostate cancer is particularly important to indicate/modulate the most appropriate therapy. Several clinical studies tried to correlate serum markers and neuroendocrine differentiation in PCa, but none of the markers investigated have been found to be sufficiently accurate to enter routine clinical practice. Our investigation has been performed by clinical staging (E-E), IHC and confocal microscopy analyses on 200 prostate biopsy provided by the Pathologic Anatomy Unit of Annunziata Hospital, Cosenza, Italy. Clinical evaluation of oncological patients was then performed before and after anti-androgen therapy. The second clinical evaluation revealed that some patients did not respond to anti-androgen therapy even if they were in the early stage of the disease; on the contrary, other patients, in an advanced stage of disease, have responded well to treatment. Hence we performed IHC and IF techniques revealing that in these cases CgA expression showed an unusual localization pattern whereas the expression of androgen receptor showed a typical expression pattern. Our results suggest that, in prostate adenocarcinoma, clinical staging, immunohistochemistry and immunofluorescence detection of both AR and CgA represent the most reliable prognostic and predictor clinical indicators.

Keywords - Prostatic cancer; early diagnosis; chromogranin A; neuro-endocrine differentiation.

I. INTRODUCTION

Prostate carcinoma (PCa) is a heterogeneous disease and represents the most commonly diagnosed malignancy of men [1]. Although many factors are involved in the progression and growth of tumoral prostatic cells, it has been known, for a long time, that androgen-androgen receptor (AR) play a pivotal role in gland carcinogens [2]. For this reason, the clinical approach is usually based on anti-androgen therapy, mainly performed in elderly subjects, whereas the same therapy is carefully weighted in young men whose quality of life should be reduced. On the other hand, with the progression of the disease, majority of patients inevitably develop androgen-independent prostate cancer, characterized by a more aggressive resistant phenotype. The molecular events that describe the androgen-refractoriness are complex and include both AR mutation or AR gene amplification, other than clonal selection and adaptive responses of tumor cells that undergo divergent differentiation processes. Accumulated evidences indicate that during prostate cancer progression the cells can undergo a trans-differentiation process to become Neuro-Endocrine (NE)-like cells, which acquire the NE phenotype and express NE markers [3,4]. NE cells represent a minor cell population in the epithelial compartment of normal prostate glands and they may play a role in regulating the growth and differentiation of normal prostate epithelia. However, in the prostate carcinoma this type of cells increases and correlates with tumor progression, poor prognosis and the hormone-refractory stage [5]. NE cells are generally androgen receptor (AR) negative, highly resistant to apoptosis, and their differentiation state is reversible. Thus, they may survive in a quiescent state and contribute to prostate cancer recurrence on dedifferentiation [5 and references therein].

Many observations, validated by in vitro studies of cultured cells [6-8] and in vivo models [9-12], have reported that, during the progression of prostate cancer cells toward the NE phenotype, the cells change morphology and express an increased level of NE growth factors that support paracrine stimuli for survival, proliferation and vasculature. The origin of NE tumor cells is discussed controversially. While some observations suggest a non-neoplastic pluripotent stem cell, in vitro studies demonstrate a transdifferentiation of exocrine tumor cells to a NE phenotype [13 and references therein]. Indeed, these cells exhibit the same genetic profile as PCa cells in tumoral lesions but not NE cells in normal tissues [13]. The NE or endocrine-paracrine cells, as previously introduced, are known to produce and secrete some potent neuro-hormones (serotonin, histamine, chromogranin A, calcitonin, neuropeptide Y, VIP, bombesin/gastrin and many other peptides) that likely modulate the functional activity of the adjacent basal and/or secretory epithelial cells [14]. All these factors can contribute to sustain the growth and progression of surrounding tumoral cells during the androgen-deprived condition [15]. Since the NE phenotype do not express AR, the androgen deprivation no longer has a therapeutic effect, rather it is able to increase and maintain high NE cell numbers and their functional activity. Understanding of the mechanisms underlying the development and function of prostate NE cells will provide useful information to

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determine whether NE cells are potential targets for novel clinical treatments.

From all this, it emerges the need for a more reliable and early diagnosis of NE differentiation in prostate malignancy in order to choose the most appropriate therapeutic approach and to modify it during the progression of the disease. Several clinical studies have tried to correlate serum markers and NE differentiation in PCa, nevertheless, to date, none of the markers investigated (including the Chromogranin A or CgA) have been found to be sufficiently accurate to enter routine clinical practice [16 and references therein].

The purpose of our study was to evaluate the possibility that the diagnosis of NE differentiation in prostate malignancy may emerges by immunohistochemical examination even in the early stages of the disease. Indeed, considering that within a tumor can coexist different histhotypes such as epithelial, exocrine, and NE, the immunohistochemical assessment of the neoplastic status becomes a crucial parameter for both diagnosis and prognosis. In a cohort of Italian men, we have examined biopsy prostatic specimens from prostate intraepithelial neoplasia (PIN), as control, and different tumor patients (from well-differentiated adenocarcinoma to a poorly-differentiated adenocarcinoma) through the analysis of two markers: AR and CgA, before and after anti-androgen therapy.

We analyzed samples from Tissue Blocks Inventory (Department of Anatomic pathology - Annunziata Hospital of Cosenza, Italy) in order to clarify the mechanisms underlying the response to anti-AR therapy with the aim of validate the prognostic role of an early CgA and AR evaluation in prostate pathology.

II. MATERIAL AND METHODS

Pathological Samples

The investigation has been performed on formalin-fixed and paraffin-embedded transrectal ultrasound guided prostate biopsy (n= 200) provided by the Pathologic Anatomy Unit (Annunziata Hospital, Cosenza, Italy). The Research Ethics Committee of Cosenza Hospital Authority approved this study. The age of the patients ranged from 55 to 86 years, the PSA values were between 7 and 35 ng/ml. All slides were reviewed by two senior pathologists (F.R. and R.DS.) in order to confirm the original diagnosis and assess histological grade. Evaluation of the tissue sections (4 µm thick) was conducted by morphological approach on Haematoxylin-Eosin (EE) stained preparations and AR and CgA expression were investigated by both immunohistochemistry (IHC) and immunofluorescence (IF) techniques.

Immunohistochemistry

Deparaffinization, rehydration and antigen unmasking were performed using a DAKO PT module (PT Link, Dako Cytomation, Denmark) according to the manufacturer's instructions (Dako Cytomation, Denmark). The enzyme immunoassay procedure was performed using a DAKO Stainer (DAKO Autostainer plus, Dako Cytomation, Denmark); the sections were incubated with the monoclonal mouse anti-human AR (1:70 in phosphate buffered saline), and the polyclonal rabbit anti- human CHA (1:50 in PBS) (both from Dako Cytomation, Denmark), followed by the secondary reagent and the polymer containing the enzyme detection Dako EnVision™ FLEX + (Dako Cytomation, Denmark).

Immunofluorescence

To evaluate the co-localization of AR and CgA, the dewaxed sections were processed according to the indirect immunofluorescence technique [17]. Sections were washed with PBS for 15 min and incubated for 10 min in a moist chamber with normal goat serum (1:50) to block non-specific sites. Sections were then incubated overnight at 4°C with the mouse monoclonal anti-human AR and the polyclonal rabbit anti- human CgA (both from Dako Cytomation, Denmark) at working dilutions of 1:50. After three washes in PBS, tetramethylrhodamine isothiocyanate conjugated γ-globulin goat anti mouse and the fluorescein isothiocyanate conjugated γ-globulin sheep anti-rabbit (both Sigma-Aldrich Chemical Co) were used as secondary antibody at dilution of 1:50 for 30 min at room temperature. Slides were rinsed again in PBS, and finally mounted. The sections were analyzed using a Leica TCS SP2 confocal laser scanning microscope (LSM).

III.RESULTS

MORPHOLOGICAL ANALYSIS

We have analyzed a total of 200 prostate biopsy, 70 of these showed a high degree of prostate intraepithelial neoplasia (PIN) and 130 presented both well-differentiated (Gleason≤7; 25%) and low-differentiated (Gleason≥8; 75%) PCa (Table 1).

TABLE I CROMOGRANIN A AND AR EXPRESSION IN PCA PATIENTS.

Neoplasia stage	*n*	*AR positivity (%)*	*CgA positivity (%)*
PIN	70	64	6
Well-differentiated PCa (Gleason≤7)	32	24	8
Low-differentiated PCa (Gleason≥8)	98	20	78

The histological evaluation in the diagnostic phase was performed on EE stained section. In non-neoplastic prostate (Fig.1A) it is possible to note the cell monolayer made by cilindric-to cubic cells that define the ducts these cells are in contact with basal membrane and are surrounded by the stromal smooth muscle fibers. In PIN sections, the parenchyma appears to be made by some group of glands showing high grade of nuclear dysplasia, where the alteration is limited to the gland (Fig.1B).

In a well-differentiated PCa, the parenchyma is characterized by epithelial neoplasia, in fact it is possible to note new formed gland that show a non-homogenous structure even

though the general architecture of gland is still maintained. Glandular cells appear hyperchromic and dysplastic (Fig.1C). In low-differentiated prostatic adenocarcinoma, the parenchyma shows epithelial neoplasia in which cells line up or appear to be organized in islet and rarely in gland. Cell features are highly atypical (Fig.1D). All 130 oncological patients were subjected to anti-androgen therapy.

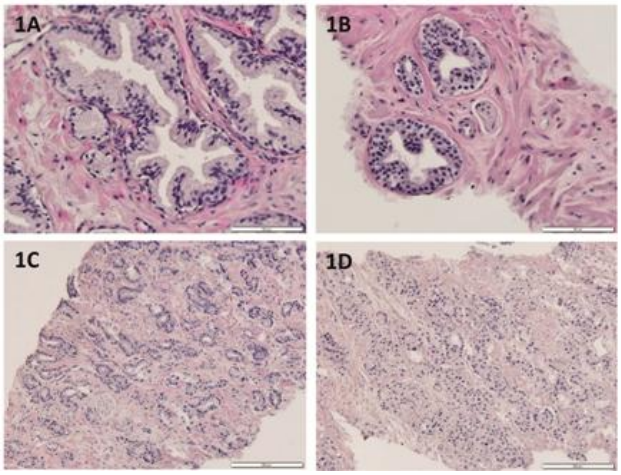

Fig. 1 Histological evaluation performed on prostate biopsy: EE stained section. A. Non-neoplastic tissue B. Prostate intraepithelial neoplasia (PIN) C. Well-differentiated adenocarcinoma D. Low-differentiated adenocarcinoma.

IHC and IF analysis

Clinical evaluation of oncological patients was performed before and after anti-androgen therapy. The second clinical evaluation revealed that some of these patients (20%) did not respond to anti-androgen therapy even if they were in the early stage of the disease; on the contrary, other patients, in an advanced stage of disease, have responded well to treatment.

For all cases we therefore have reconsidered specimens used for first evaluation (i.e. E-E on biopsy); on these we performed the immunohistochemical localisation of both androgen receptor (AR) and chromogranin (CgA) (Fig. 2A-F). We revealed that in most cases (80%) the AR and CgA localisation show a typical pattern. In both non neoplastic and PIN prostatic regions CgA is poorly expressed: weak signals could be appreciated in few epithelial cells that form the duct (Fig 2A). Similarly, in a well-differentiated PCa the intensity of staining is low and it is localised in the epithelial cells that line the duct (Fig. 2B). On the contrary, in the low-differentiated PCa an intense immunopositivity for CgA could be detected, mainly localized at epithelial level (Fig 2C).

The evaluation of AR expression showed an intense immunelabeling at stromal and epithelial levels of prostatic ducts in both non neoplastic and PIN prostatic regions (Fig. 2D). The AR maintains the same pattern of expression in a well-differentiated PCa (Fig. 2E) while the intensity of the staining strongly decreases in a low-differentiated PCa (Fig. 2F).

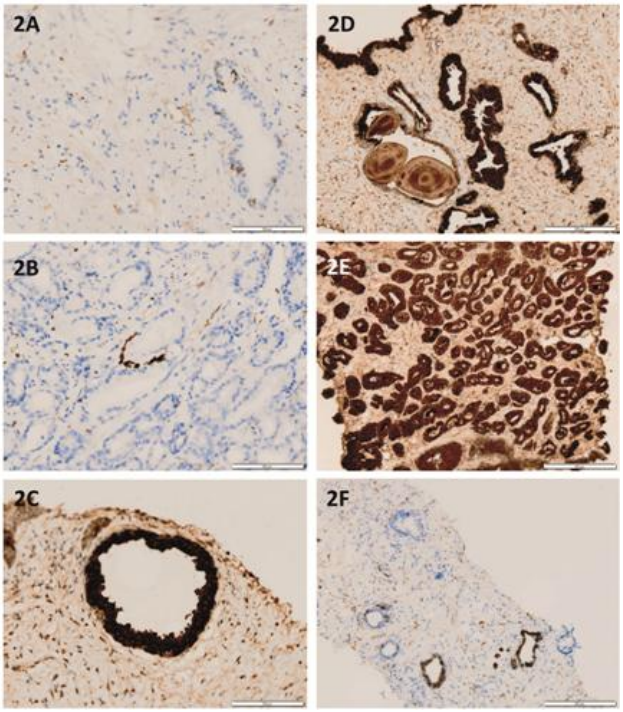

Fig. 2 Immunolocalisation (IHC) of chromogranin A (CgA) an androgen receptor (AR). A. CgA localization in non-neoplastic tissue B. CgA localization in well-differentiated adenocarcinoma C. CgA localization in low-differentiated adenocarcinoma D. AR localization in non-neoplastic tissue E. AR localization in well-differentiated adenocarcinoma F. AR localization in well-differentiated adenocarcinoma.

We also performed co-localisation analysis of AR and CgA: in non neoplastic and PIN prostatic samples the AR is evenly distributed and well represented, while CgA is nearly absent (Fig. 3A). In particular, a high positivity for the AR was detected in a well-differentiated PCa, at both stromal level and epithelial cells of the ducts, while the CgA expression was modest, especially in the ducts and very limited in the stroma (Fig. 3B). In the case of poorly differentiated PCa, we revealed a remarkable increase in CgA positivity and a marked decrease in AR immunolabelling (Fig. 3C).

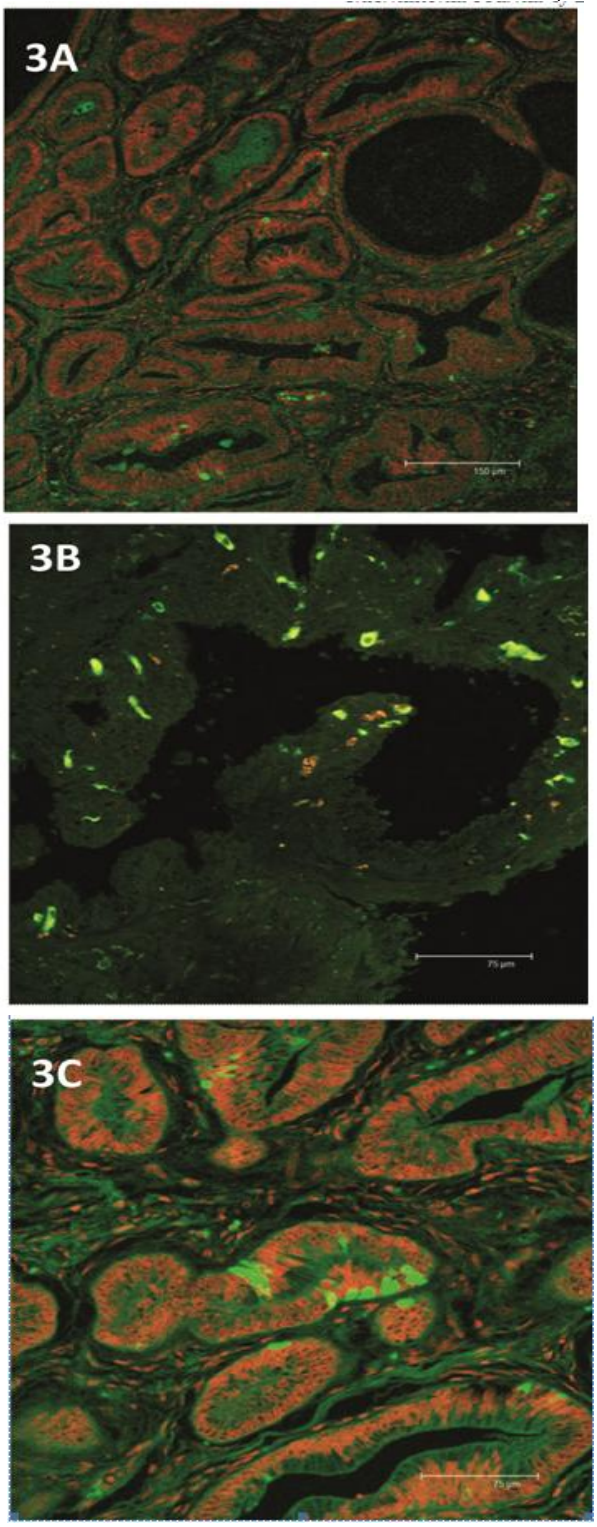

Fig. 3 Co-localisation analysis of CgA and AR.

A. CgA (FITC) and AR (TRITC) localization in non-neoplastic tissue B. CgA (FITC) and AR (TRITC) localization in well-differentiated adenocarcinoma C. CgA (FITC) and AR (TRITC) localization in low-differentiated adenocarcinoma.

In table 1 we have summarized the results from immunohistochemical analysis: the chromogranin expression in PCa varied from CgA negativity/low-positivity to CgA high-positivity, depending on the progression (well-differentiated or low-differentiated PCa) and the degree of disease; it is also evident that the increase of CgA positivity is inversely correlated with the AR expression.

Interestingly, a percentage of cases (20%) presented an opposite trend, relatively to neuroendocrine marker expression: we found a significant positive CgA in the initial stage of the disease (well-differentiated PCa), and a low positivity to CgA in advanced pathology (low-differentiated PCa).

However, the expression of the androgen receptor remains almost unchanged, showing only a small percentage of positivity compared to the cases of well and poorly differentiated carcinoma analyzed in our sample. By analyzing these cases after anti-androgen therapy, it was observed the following clinical trend: patients with PCa at the initial stage (well-differentiated), but with high CgA expression, did not get benefits from therapy (80% of the sample showed higher levels of neuroendocrine positivity and then a progression to neoplasia, while 20% died during therapy); while patients with a more advanced stage of PCa (low-differentiated), but with few or no NE markers, have benefited from therapy (90% of the sample did not show an increase in neuroendocrine positivity and then got control of the tumor, while only 10% showed a more high NE positivity and then a progression to NE tumors).

IV. DISCUSSION

It is widely known that prostate gland, from the stage of benign glandular hyperplasia, can unfortunately undergo morphological changes up to acquire the tumoral phenotype and after long-term anti-androgen therapy it becomes enriched for neuroendocrine cells [18,19]. Considering that, within a single tumor may coexist different oncotypes (exocrine, epithelial and neuroendocrine), the immunohistochemical analysis of the neoplastic state, before starting the therapy, gets a fundamental parameter both from a diagnostic and prognostic point of view.

The hyperactivation of NE phenotype, following long-term anti-androgen treatment, is a type of response that should be well assessed and rightly considered as early, since these cells prevalently provide proliferative stimuli to surrounding cancer cells. In the development of adenocarcinoma it must also keep in mind the close relationship between the epithelial compartment and the neuroendocrine cell type, as the first compartment is almost dependent on androgens, while the second one is no longer dependent. Indeed, It is well-known that prostate cancer-associated neuroendocrine cells do not express androgen receptor [20, 21], they resist apoptosis [22], so that patients with this oncotype are addressed towards an adverse clinical outcome .

Based on the results obtained in normal prostate samples, it can be argued that AR is uniformly present in epithelial cells of the prostatic ducts and partially present in the stroma, whereas the expression of CgA is almost absent. This pattern of expression is almost confirmed in a well-differentiated tumors.

An interesting evaluation of the biopsies was provided by confocal microscopy, which highlighted the dynamism of prostate gland in a process of neuroendocrine differentiation. Indeed, our results have demonstrated during the progression of neoplasia many morphological changes, related to the different relationship between the AR and CgA, rather than their presence and/or absence. For diagnosis, the predictive value of these markers becomes very effective and, above all, how they relate. In fact, in samples that show progressively PIN, from a well differentiated adenocarcinoma up to that poorly differentiated adenocarcinoma, the fluorescent AR signal is weak and in some areas absent; while the CgA becomes consistent. The expression of these markers are well associated with the clinical oncotype and once again confirm their prognostic value.

In contrast to what has recently described on circulating CgA levels, which are not significant predictors of poorly differentiated CaP on initial prostate biopsy, this marker assumes particular relevance and clinical validity through IHC/IF detections, as here reported [16]. Indeed, in a low-differentiated PCa, we have revealed an intense CgA positivity prevalently localized at the epithelial compartment.

However, in our cases, we found ourselves in front a percentage of samples that, although small, had abnormal characteristics and dissimilar from that above reported.

Surprisingly, in a limited series of 20% of well-differentiated carcinoma, the fluorescent AR signal appears very weak and in some areas absent, while the CgA expression becomes consistent. These features are predictors (cohort of patients) to a worsening prognosis, even if we are dealing with well-differentiated carcinoma. In these cases, an early diagnosis is crucial.

The follow-up study, referred to this cohort of patients and carried out before and after anti-androgen therapy, revealed that patients at the first stage, with moderate or high positivity for NE marker, did not benefit of the therapy, as part of them died . This addresses the potential adverse impact of CgA in a well differentiated tumors. On the contrary, in patients with a low- differentiated tumors, anti-hormonal treatment kept the low positivity for CgA. Likely, the hormonal withdrawal (ablation), in this subgroup of patients of advanced stage, may help to maintain the low neuroendocrine positivity and, probably, can be useful to control tumor growth. In fact, only in about 10% of the cases there was a moderate increase of CgA.

V. CONCLUSIONS

This longitudinal study, conducted in a rich cohort of patients has provided useful and detailed information for diagnostic purposes. First, we saw that the simple EE-stained preparation is not always consistent with the real picture. Certainly, the morphological assessment together with IHC/IF profile represent the "gold" standard to deliver a better differential diagnosis. We can state that in the evolution of prostate cancer, there is a phase in which the prostate cells assume a 'hybrid' morphology, which will determine neuroendocrine connotations with unfavorable prognosis. Taken together, our results suggest that, in prostatic adenocarcinoma, clinical staging, IHC and, more recently, confocal microscopy analyses, for both AR and CgA, represent the most reliable prognostic and predictor clinical indicators

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