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Activation of Epidermal Growth Factor Receptor (pEGFR) and Expression of Argentophilic Nucleolus Organizer Regions (AgNOR) in Seminomas

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Authors' contributions

This work was carried out in collaboration between all authors. Author FG did the experimental studies and immunohistochemistry/AgNOR. Author AER managed the experimental studies and wrote the manuscript. Author AA anchored the experimental studies and AgNOR. Author MAA managed pathological diagnosis of the smears. Author LBD completed the pathological diagnosis of the smears and literature search. Author LJ managed pathological diagnosis of the smears and wrote the manuscript. Author GM carried out experimental studies and statistical analysis. Author LAP did the study design and review of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Activation of epidermal growth factor receptor (p-EGFR) is one of the triggers in the development of many malignant tumors, and the measurement of Argentophilic Nucleolus Organizer Regions (AgNOR) area is used as a marker of tumor proliferation. Alpha actin smooth muscle (αASM) of

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peritubular cells (PT) and Vimentin expression are modified during the expansion of the CIS /intratubular germ cell neoplasia (ITGCN) to solid seminoma. We evaluated the expression pEGFR, the AgNOR areas, the expression of αASM in PT and the Vimentin in neoplastic cells from CIS/ ITGCN to solid seminoma.

28 formalin-fixed paraffin-embedded archival tissue blocks of Seminomas from the Departments of Pathology of Clinical Hospital and Ramos Mejía Hospital (CABA, Argentina) were used in this study. pEGFR was expressed in CIS/ ITGCN with membranous pattern (8/9), switching to a cytoplasmic pattern in the solid seminoma (15/19). AgNOR areas of atypical gonocytes were increasing from CIS (3.5±0.3 μ^2) to intratubular seminoma (3.8±0.4μ^2) (p<.5), until solid seminoma (5.3±0.7 μ^2) (p<.01). Increase of AgNOR areas is proportional to the expression of pEGFR.

Conclusions: pEGFR was expressed in CIS/ ITGCN with membranous pattern, switching to a cytoplasmic pattern in the solid seminoma. The activation of this receptor could be the first step in the mitogenic signal transduction.

AgNOR areas complement diagnosis of testicular CIS, but not differentiate between CIS and intratubular seminoma. The value of AgNOR areas is maximum in the invasive seminoma, and statistically different from intratubular seminoma. The intensity of αASM in PT decreased as the tumor progressed and Vimentin was negative in neoplastic cells.

Keywords: Seminoma; phosphorylated epidermal growth factor receptor; AgNOR; Alpha actin smooth muscle; vimentin.

1. INTRODUCTION

It is considered that the seminoma and non seminoma testicular tumors have origin from a common precursor: CIS /intratubular germ cell neoplasia (ITGCN) [1], except the pediatric testicular germinal tumors (yolk sac tumours and mature teratomas) and the rare spermatocytic seminoma occurring in elderly men [2]. CIS /intratubular germ cell neoplasia (ITGCN) is believed to arise from failure of normal maturation of fetal germ cells from gonocytes into pre-spermatogonia [3-5] The CIS expresses useful markers for identification in cases of inconclusive morphology, as placental-like alkaline phosphatase [6] or Ki-67 [7]. Over the years, new markers were identified: TRA-1-60 [8], M2A [9], c-KIT [10], which are abundant in CIS, in normal fetal and infantile germ cells but undetectable in the normal adult testis, These observations support the hypothesis of the prenatal origin of CIS. Underlining this hypothesis, OCT-4, AP-2γ and NANOG are detected in early fetal gonocytes and CIS, decreasing expression while gonocytes differentiate to spermatogonia [11].

Sertoli cells accompanying atypical gonocytes express vimentin in the CIS; This marker disappears together with the Sertoli cells as tumor progression occurs [12].

Argentophilic Nucleolus Organizer Regions (AgNOR) express the replication capacity of a cell or epithelium; the number of particles or the surface occupied in the nucleus is related to the mitosis velocity in many tissues [13,14].

AgNOR was useful in confirming the intratubular testicular neoplasms, with similar results to those obtained using placental-like alkaline phosphatase [15].

Growth factors and their receptors have been found to play an important role in the pathogenesis of several malignancies. Epidermal growth factor receptor (EGFR) is a plasma membrane glycoprotein composed of an extracellular ligand-binding domain, a transmembrane lipophilic segment, and an intracellular protein kinase domain [16].

After binding of ligand, the EGFR dimerization leads to activation of a cascade of biochemical responses that are involved in the mitogenic signal transduction of normal as well as malignant cells [17,18]. These pathways are, principally, MAPK (Mitogen-activated Protein Kinase), Akt (Protein Kinase B) and JMK(c-Jun NH2-terminal kinase).

EGFR expression is elevated in many epitelial tumors and coexpression of high levels of EGFR and its ligands lead to a malignant trasformation. Therefore, EGFR may be target for anticancer therapies [19].

The environment or niche surrounding the gonocytes (Sertoli cells, peritubular myoid cells (PT), the extracellular matrix and Leydig cells)

also influences the development and expansion of seminoma.

In humans, PT cells have been reported to be a secondary source of growth factors (as GDNF: glial cell line-derived neurotrophic factor) that influence spermatogonial stem cell (SSC) proliferation and self-renewal. In CIS, PT cells might respond to CIS-derived factors, including PDGF and TGF-b, increasing their proliferation and secretory activity [20].

The objectives of this paper are to evaluate the expression of phosphoritated EGFR (pEGFR) from CIS/ ITGCN to solid seminoma in biopsies of this tumor, as the first step in the mitogenic signal transduction. Besides, we compare the AgNOR areas in the different stages of seminoma, especially in the confirmation of CIS, when the morphology is unclear, and the expression of Vimentin and alpha actin smooth muscle (αASM) in PT cells, indicators of the progression of neoplasia.

2. MATERIALS AND METHODS

28 formalin-fixed paraffin-embedded archival tissue blocks of Seminomas from the Department of Pathology of two hospitals were used in this study. Of them, 9 were CIS/ITGCN (Table 1).

Table 1. Main characteristics of the patients with seminoma

	CIS/ ITGCN (9)	Solid seminoma (19)
Age (years)	16-26	24-35
History of cryptorchidism	6/9	11/19
Subfertility	2/9*	5/19

(5 cases were not studied)*

2.1 Innmunohistochemistry

3-5 µm thick tissue sections (about 15 of each smear) were deparaffinized in Xylene in two steps: 30 minutes at 56℃ and 20 minutes at room temperature. Then were rehydrated through graded concentrations of ethylic alcohol and placed in citrate buffer pH 6,0 for antigen retrieval during 30 minutes at 96-98℃. Then, the slides were cooled in the same buffer for 20 minutes and placed in phosphate buffer PBS pH 7.2. Endogenous peroxidase was blocked with 3% H_2O_2 in methanol. The samples were incubated with several primary antibodies: Anti-phospho-EGFR (Tyr1173) Rabbit monoclonal 1:50 (EMD Millipore Corporation, Temecula, California, USA), Anti-Human Vimentin mouse monoclonal 1:200 (EMD Millipore Corporation, Temecula, California, USA) and Anti-alpha actin smooth muscle 1:200 mouse monoclonal (Cell Marque, Rocklin, California, USA). All incubations were carried out overnight. Then, the slides were incubated successively with biotinilated-secondary antibody, streptavidin-peroxidase (Mouse/Rabbit PolyScan HRP/DAB Detection System Cell Marque, Rocklin, USA) and hydrogen peroxide with diaminobenzidine. Development of brown colour showed the presence of the corresponding antigen. In small number of biopsies was used biotinilated-antibody, streptavidin-alcaline phosphatise and Fast Red TR. (Sigma-Aldrich, St Louis MO, USA). The development of red colour was interpreted as positive.

2.2 AgNOR Assay

After deparaffinization, the samples were immersed in 96° alcohol with acetic acid, and were incubated in the dark for 25 min with a mixture of silver nitrate 50% (W/V) and (2:1) 1% gelatin (W/V): 1% formic acid (V/V). After washing with deionized water and 1% sodium thiosulfate (W/V), the slides were dehydrated and mounted with Canada balsam. The average AgNOR areas were determined by image cytometry (Image Pro-Plus, Version 4-5, Media Cybernetics, Bethesda MD) using the oil immersion objective and selecting 100 cells in each smear [21].

2.3 Statistical Analysis

Statistical analysis was performed with SPSS 16.0 software (SPSS Inc., Chicago, USA). A *p-value* less than .05 was considered statistically significant.

2.4 Ethics Statement

This is a retrospective research with archived biopsies, anonymized for the use in this study. Thus no informed consent was needed. (National Law on Protection of Personal Data, No. 25326 - Argentina). This study was approved by the Institutional Review Board at the Clinical Hospital-University of Buenos Aires.

The researchers respect the Declaration of Helsinki in its latest version (World Medical Association Declaration of Helsinki 2013).

3. RESULTS

pEGFR was positive in atypical gonocytes in 8/9 CIS, showing a membranous (peripheric) pattern. Some cells showed a cytoplasmic expression of the receptor. The tubules with CIS close to the areas of solid seminoma also expressed the membranous/cytoplasmic pattern in12/19 cases of seminoma.

The seminomas showed membranous/ cytoplasmic (intratubular) or cytoplasmic expression of pEGFR in 15/19 cases.

Sertoli cells showed a slight expression of pEGFR in both tubules with CIS or intrabular seminoma (Fig. 1).

AgNOR areas of atypical gonocytes were increasing from CIS (3.5±0.3 μ^2), intratubular seminoma (3.8±0.4 μ^2) until solid seminoma (5.3±0.7 μ^2) ($p< .5$ between CIS/intratubular seminoma; $p<.01$ between intratubular/solid seminoma).

The AgNOR particles showed two patterns in solid semiomas: Single and round, or multiple and irregularly shaped.

The AgNOR particles in CIS were multiple and irregularly shaped, making it difficult to measure the area.

Some cells were used as controls in the same biopsies. AgNOR areas were low in spermatogonia (0.11 μ^2), spermatocytes (0.34 μ^2) and epithelial cells of epididymis (0.65 μ^2). Sertoli cells reached AgNOR areas of 1.58 μ^2, showing unique and round particles (Fig. 2).

PT cells surrounding the tubules with normal spermatogenesis strongly expressed αASM. Its intensity was declining as the tumor progressed, almost disappearing in the nests of solid seminoma. Interestingly, PT cells remain in many seminomas, but failed to express αASM (Fig. 3).

Vimentin was positive in Sertoli cells and negative in atypical gonocytes in 9/9 CIS. In invasive seminoma, only stromal areas showed reactivity (Fig. 4).

4. DISCUSSION

Gene expression studies showed that CIS cells resemble embryonic stem cells. Atypical gonocytes express embryonic factors as OCT-3/4 and AP-2γ, associated with undifferentiated and pluripotency of germ cell tumors. Loss of E-cadherin, a key component of adhesion junctions, is characteristic of EMT and it is associated with tumor cell invasion [22]. Expression of E-cadherin was low or absent in the majority of the CIS and seminomas. However, EMT does not seem to occur during

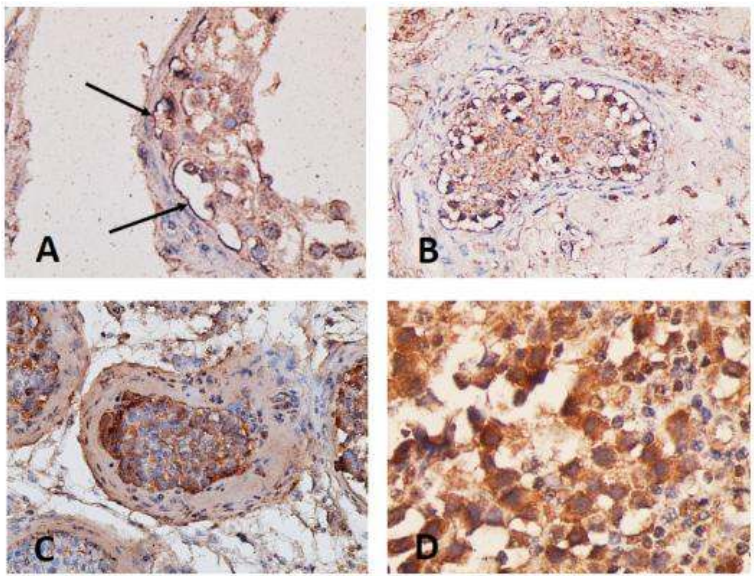

Fig. 1. Expression of p-EGFR

A: p-EGFR with membranous pattern in CIS (arrows) B: p-EGFR in CIS/intratubular seminoma)
C: p-EGFR with membranous/cytoplasmic pattern in tubular seminoma
D: p-EGFR with cytoplasmic pattern in solid seminoma (Immunohistochemistry. B andC 400x – A and D 1000x-counterstaining with hematoxylin)

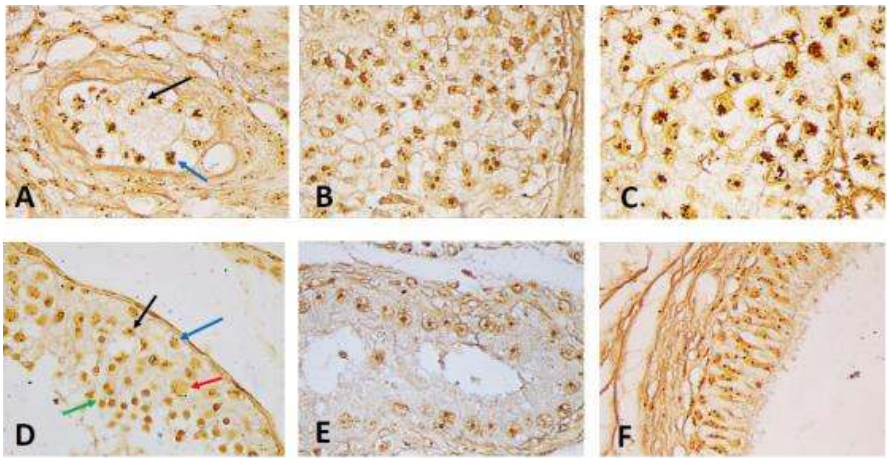

Fig. 2. Expression of AgNOR

A: AgNOR in CIS. Sertoli cells show a single round dot (black arrow), and atypical gonocytes show many irregular spots, with larger area than the Sertoli (blue arrow). B: Solid seminoma with round AgNOR pattern. C: Solid seminoma with multiple and irregular AgNOR spots. D: AgNOR expression in a tubule with normal spermatogenesis. Sertoli (black arrow), Spermatogonia (blue arrow), Spermatocyte (red arrow) and Spermatide (green arrow) E: AgNOR in SCO F: AgNOR in epididymis. (all pictures in 400x)

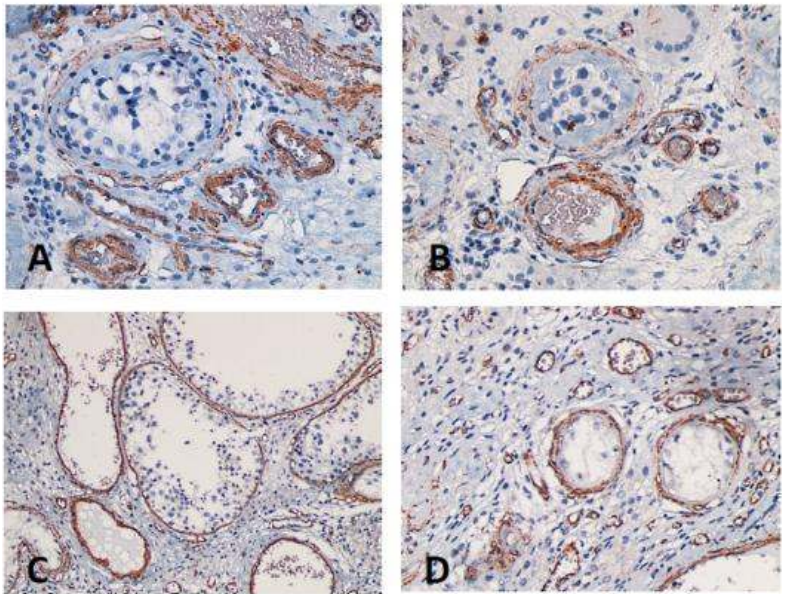

Fig. 3. Alpha actin smooth muscle (αASM)

A: CIS: αASM is weak and discontinuous in the PT; compare the expression of this marker in the endothelium of nearby capillaries. B: Intratubular seminoma. αASM shows a similar pattern than that of CIS in PT; compare with the capillary below the tubule. C: Strong and continuous expression of αASM in the PT surrounding a normal tubule. D: Strong and continuous expression of αASM in the PT surrounding tubules with SCO. (Immunohistochemistry, 200x - counterstaining with hematoxylin)

the progression of seminomas [12]. EMT is a phenomenon known in the progression of many cancers, where neoplastic epithelial cells change their genotype / phenotype to mesenchymal cells. During the EMT, mesenchymal markers such as vimentin and αASM are expressed, while epithelial markers such as E-cadherin and cytokeratin disappear. This does not happen in seminomas, because the epithelium of the seminiferous tubules has a double embryological origin: it is the only epithelium consists of a set of mesodermal cells (Sertoli) and a set of cells

derived from the yolk sac. These last cells differentiate to pre-spermatogonia, do not arise from any of the three sheets of the embryo (ectoderm, endoderm or mesoderm) and express embryonic markers. During the invasion process, the seminoma cells do not express neither αASM nor vimentin (except very few cells expressing this last marker). Therefore, although the epithelium of seminiferous tubules is considered as such, malignant germ cell does not meet the postulates of EMT.

The histological diagnosis of testicular CIS presents difficulties. Seminiferous tubules with atypical gonocytes can be very scarce, and mixed with tubules with signs of gonadal dysgenesis. Expression of AgNOR is a useful complement to the correct diagnosis of CIS, as the NOR areas of atypical gonocytes exceed the value of the NOR areas of Sertoli cells. Added to this, the pattern of AgNOR particles in atypical gonocytes is irregular, in contrast to that of Sertoli cells, characterized by unique and round particles (observe AgNOR particles in Sertoli Cells Only (SCO), Fig. 2E). On the other hand, the difference between NOR areas of atypical gonocytes and spermatogonia is even greater. However, the difference between the areas of AgNOR between CIS and intratubular seminoma is not statistically significant. In this case, the morphology (H & E) retains an important value.

Finally, as the germ tumor progresses from intratubular seminoma to solid seminoma, AgNOR areas increase with difference statistically significant.

Vimentin is a protein that forms the cytoskeleton of mesenchymal cells, therefore is expressed in Sertoli cells from tubules with CIS, SCO tubules and tubules with normal spermatogenesis. Immunohistochemistry for vimentin is helpful in CIS because the contrast between atypical gonocytes negativity and positivity of Sertoli cells helps identify the firsts (Fig. 4B).

PT cells have contractile capacity, because it contains actin and myosin. They form a barrier that helps to separate seminiferous tubules of interstice.

Using an antibody against αASM, it was demonstrated strong expression of this protein in PT surrounding normal tubules and the decreasing as the germinal tumor progresses, disappearing in the solid seminoma. The loss of αASM involves changing of phenotype from PT to fibroblast with a loss of elasticity of the tubular wall [23].

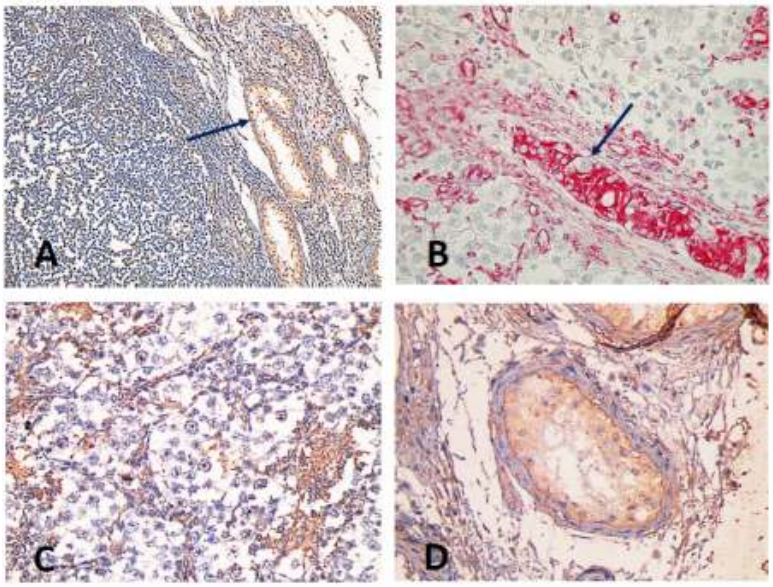

Fig. 4. Expression of Vimentin

A: A tubule with CIS shows expression of Vimentin in Sertoli cells (arrow). To the left of the picture, an area of solid seminoma is negative for the marker B: The arrow points an atypical gonocyte negative for Vimentin. The Sertoli cells show strong expresion for this marker C: Solid seminoma. The neoplastic cells not express Vimentin, which is only detected in the remains of stroma D: Sertoli cells expressing Vimentin in a tubule with SCO. (Immunohistochemistry; A 100x – B,C and D 400x - counterstaining with hematoxylin)

Seminoma expands as the tubular walls lose elasticity, filling and widening tubules (intratubular neoplasia) until the disappearance of the barrier produces output and dispersion of neoplastic cells (solid seminoma).

Activation of epidermal growth factor receptor (p-EGFR) is one of the triggers in the development of many malignant tumors. The autophosphorylation elicits activation of proteins that iniciate several signal transduction cascades (MAPK, Akt, JNK) leading to cell proliferation.

Expression of the EGFR ligand TGF-α and the activated form of EGFR (p-EGFR) was found in 36 and 27% of EGFR positive, non seminomatous GCTs, respectively [24]. Overexpression of the EGFR protein was detected in 28% of seminomas (27% in the pure-form and 29% in the mixed-form). Samples were analyzed for expression of EGFR protein and EGFR gene amplification by immunohistochemistry and fluorescence in situ hybridization [25].

In our series, testicular CIS shows a peripheral pattern (membranous) on p-EGFR expression of atypical gonocytes, while the solid seminoma shows cytoplasmic expression of p-EGFR. A slight expression of this marker was also detected in Sertoli cells. Recently, it was identified a nonclassical mechanism of testosterone signaling in cultured rat Sertoli cells. Src, activated by the binding of testosterone to their receptor, activates the epidermal growth factor receptor, which results in the phosphorylation and activation of the ERK MAPK and the cAMP response element-binding protein transcription factor. Ift his route was active in humans, would explain the slight expression of pEGFR in Sertoli cells in our biopsies [26].

So far as we know, this is the first communication on p-EGFR expression in seminomas. The low number of samples of our population somewhat limits the conclusions of our study. Today we continue with investigations especially in new cases of CIS.

We are developing experiments to search if some of the routes related to this receptor (MAPK, Akt and JMK) are activated in these tumors.

5. CONCLUSIONS

pEGFR was expressed in CIS/ ITGCN with membranous pattern, switching to a cytoplasmic pattern in the solid seminoma. Presumably, this could be the first step in the mitogenic signal transduction.

AgNOR areas complement diagnosis of testicular CIS, but not differentiate between CIS and intratubular seminoma. The value of AgNOR areas is maximum in the invasive seminoma, and statistically different from intratubular seminoma. Increase of AgNOR areas is proportional to the expression of pEGFR.

Vimentin and αASM in PT cells, are indicators of the progression of neoplasia.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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