 doi:10.20944/preprints202103.0273.v1

Original paper

Overexpression of p75^{NTR} in Testicular Germ Cell Tumors: a New Biomarker of Cancer Differentiation?

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Abstract: Several studies have demonstrated that the p75^{NTR} low-affinity receptor of Nerve Growth Factor (NGF), is produced in abnormally large amounts in several human cancer types. However, the role of p75^{NTR} varies substantially depending on cell context, so that a dual role of this receptor protein in tumor cell survival and invasion, as well as cell death, has been supported in recent studies. Herein we explored for the first time the expression of p75^{NTR} in human specimens (nr=40) from testicular germ cell tumors (TGCTs), mostly seminomas. Nuclear overexpression of p75^{NTR} was detected by immunohistochemistry in tumor tissue as compared to normal tissue, whereas neither NGF nor its high-affinity TrkA receptor was detected. An increased nuclear staining of phospho-JNK, belonging to the p75^{NTR} signaling pathway, and its pro-apoptotic target gene, p53, was concomitantly observed. Interestingly, our analysis revealed that decreased expression frequency of p75^{NTR}, p-JNK, and p53 was related to staging progression, thus suggesting that p75^{NTR} may represent a specific marker of differentiation in TGCTs.

Keywords: testicular germ cell tumors (TGCTs); human seminoma; p75 neurotrophin receptor (p75^{NTR}); p75^{NTR} -signaling.

1. Introduction

Testicular germ cell tumors (TGCTs) are the most common tumors in adolescent and young men, accounting for almost all testicular cancers, and their incidence is rising especially among Caucasians [1]. TGCTs are divided into seminomatous germ cell tumors (SGCT) and non-seminomatous germ cell tumors (NSGCT), the latter including undifferentiated (embryonal carcinoma) or differentiated (teratoma, yolk sac tumor, and choriocarcinoma) tumors [2]. TGCTs have exceptional cure rates compared to other tumors, as 95% of affected men survive over 5 years [3]. Therefore, accurate staging and correct histopathological recognition of these tumors are critical for targeted therapy and optimal outcomes. Recent proteomic analysis of TGCTs identified novel proteins that might be used to better understand the molecular mechanism(s) involved in TGCTs [4]. As reported elsewhere [5], the histogenesis of TGCTs is complex, and it is thought that TGCTs might develop from premalignant intratubular germ cell neoplasia that progresses toward

 doi:10.20944/preprints202103.0273.v1

invasive seminoma and/or nonseminoma after puberty, when cells start to proliferate under the influence of hormones [6]. It is believed that TGCTs may arise from the failure of normal maturation of gonocytes, although, to date, the exact molecular derangements underlying this transformation are not clearly understood, even if the most common genetic finding is the gain of genetic material from chromosome 12p [7]. Since mammalian spermatogenesis is an intricate sequential process of germ cell differentiation from primordial germ cells or spermatogonial stem cells to functional haploid sperm that occurs via a complex interaction between germ and somatic cells, a better knowledge of the regulatory control of spermatogenesis should provide crucial insights into the occurrence and features of TGCTs [8–11]. Studies in human and animal models have demonstrated that during testicular development, the neurotrophins, a family of polypeptide growth factors, and their receptors, are expressed in germ cells throughout their development and also in somatic cells, suggesting that the activation of their receptors may be important in testicular development and spermatogenesis [12–15].

Among neurotrophins, Nerve Growth Factor (NGF) and its high-affinity receptor, TrKA, before birth, exert an important role in seminiferous cord formation, germ cell differentiation, and Sertoli cell viability; after birth, they are involved in sperm motility and acrosome reaction. Fewer data are available on the potential function of the low-affinity NGF receptor $p75^{NTR}$, a member of the tumor necrosis receptor superfamily, which seems to have important implications in early testicular development [16]. Interestingly, immunohistochemistry studies have demonstrated that NGF/TrkA/ $p75^{NTR}$ are expressed in human breast, ovarian, and prostatic cancers, suggesting that they may represent new diagnostic markers. Concomitantly, *in vitro* and *in vivo* cancer models showed that NGF is involved in cancer cell progression, invasion, and chemoresistance, underlying that the NGF axis may represent a new therapeutic target in these tumors [17].

To our knowledge, there are no studies that have investigated the expression pattern of NGF/TrkA and $p75^{NTR}$ in testicular cancer. To better understand the potential implications relevant for the NGF signaling pathway in TGCTs, the objective of the current study was to explore the expression of NGF and its receptors particularly in human testicular seminoma, the most common histological type of TGCTs.

2. Results

2.1 Clinical characteristics of collected samples

Forty samples of primary TGCTs were collected. The median age of the 40 patients was 38 years (ranging from 25 to 51 years), and all patients were Caucasian.

More than half of the collected specimens presented with stage I neoplasms (67.5%) and the remaining were among stages II (17.5%), III (12.5%), and IV (2.5%), respectively. Seminoma was the most frequent histological type (65%), while embryonal carcinoma and mixed GCT were less represented (27.5% and 7.5%, respectively). Control testicular tissues were obtained from 4 male patients (aged 31 and 44 years), showing testes with a like-sarcoidosis granulomatous lesion.

2.2 Immunohistochemical of p75NTR, NGF and TrKA expression in TGCC

Immunohistochemical analysis revealed a strong nuclear immunoreactivity of $p75^{NTR}$ in the tumor sections compared to control tissue sections (Figure 1 G-H), whereas neither NGF nor TrKA staining was observed in the tumoral tissue (Figure 1 C-F).

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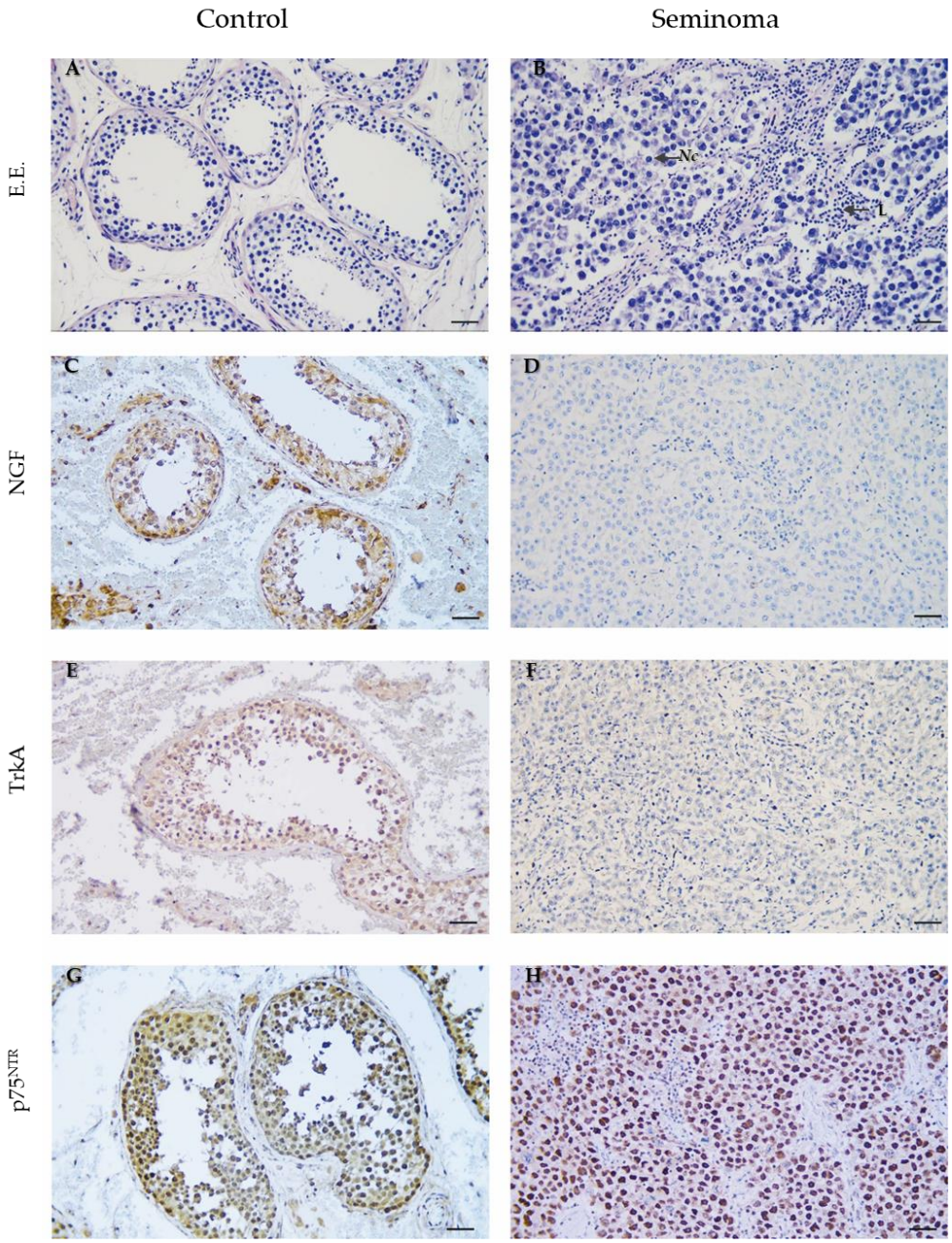

Figure 1. Morphology (A, B) and Immunohistochemical expression of NGF (B, C), TrkA (D, E), and $p75^{NTR}$ (F, G) in control testis and seminomas. (Nc) neoplastic cells; (L) lymphocytes. Scale bars: 25 μm.

The immunohistochemical expression (Table 1) was evaluated using the "Allred Score" (percent of positive cells) and intensity score (negative/weak/moderate/intense).

Table 1. Immunostaining scores (Allred score median) of NGF, TrkA, and $p75^{NTR}$ in seminoma samples. Immunostained slides scores as follows: Total score = Proposition score + Intensity score (range 0-8).

Marker	Control	Seminoma
NGF	3	0
TrkA	2	0
$p75^{NTR}$	4	8 §

§ $p < 0.0001$ (one-way ANOVA test) versus control.

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Cutoffs for positivity of $p75^{NTR}$ marker were defined based on the area under the ROC curve, considering the sum of the score and the occurrence of clinical events (Table 2).

Table 2. $p75^{NTR}$ cutoff based on the area under the ROC curve.

Marker	Cutoff	Sensitivity (%)	Specificity (%)	PPV	NPV	AUC (95% CI)
$p75^{NTR}$	≥ 7	67.8%	59.0%	21.4%	76.0%	0.80 (0.75-0.98)

AUC – Area under ROC curve; CI - Confidence interval; PPV – Positive predictive value; NPV – Negative predictive value

Concomitantly, we explored whether the observed overexpression of $p75^{NTR}$ was accompanied by a modulation of its downstream signaling JNK in seminoma tissue. Interestingly, in tumoral tissue, we found a decreased expression of total JNK (Figure 2 A-B; Table 3), with an increased expression of p-JNK as compared to control (Figure 2 C-D; Table 3).

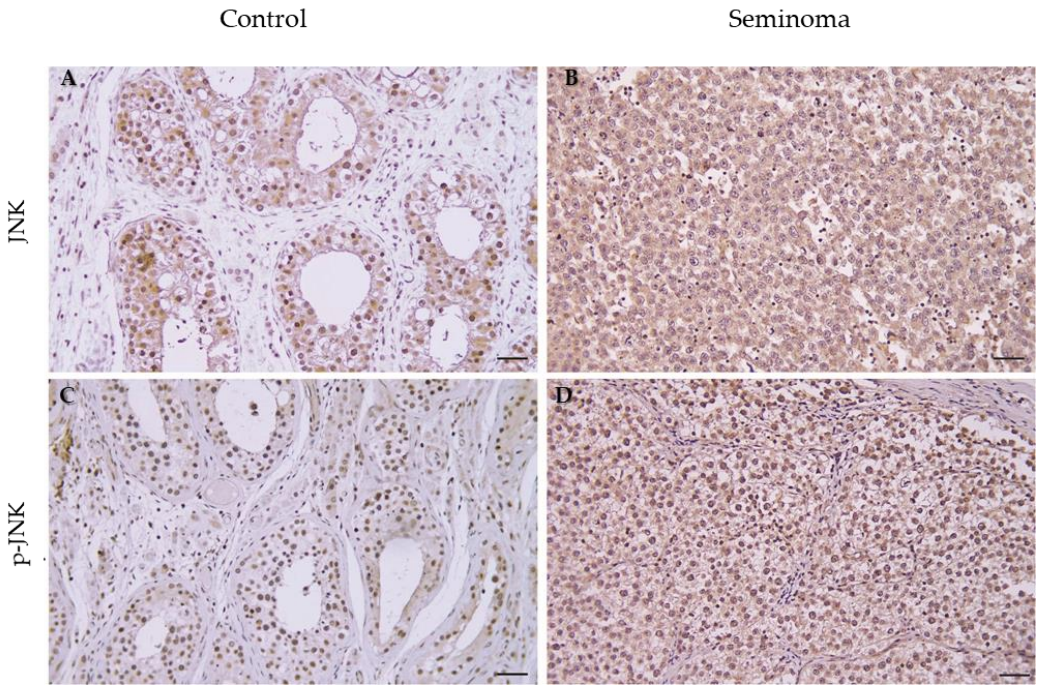

Figure 2. Immunolocalization of JNK (A, B) and p-JNK (C, D) in control testis and seminomas. Scale bars: 25 μm.

2.3 Immunohistochemical of p53 in TGCC

Immunohistochemical analysis revealed a strong nuclear immunoreactivity of p53 in the tumor sections compared to control tissue sections (Figure 3, Table 3).

Table 3. Immunostaining scores (Allred score median) of JNK, p-JNK, and p53 in human seminoma samples. Immunostained slides scores as follows: Total score= Proposition score+ Intensity score (range 0-8). * P< 0,001 (one-way ANOVA test) versus control; **P< 0,005 (one-way ANOVA test) versus control.

Marker	Control	Seminoma
JNK	6	4**
p-JNK	5	6*
P53	2	7**

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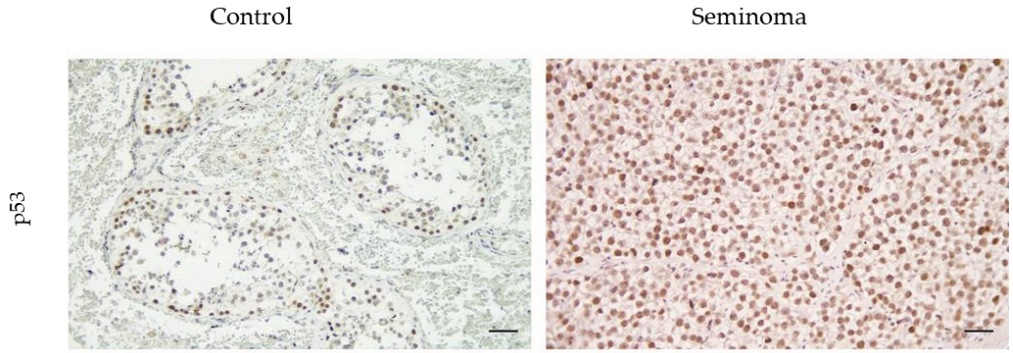
Figure 3. Immunolocalization of p53 in control testis and seminomas. Scale bars: 25 μm.



2.4 Validation of TMA method

Analysis of agreement between TMA and whole tissue sections showed an accuracy for TMA method of 80% for NGF, 98% for TrkA, and 78% for $p75^{NTR}$.

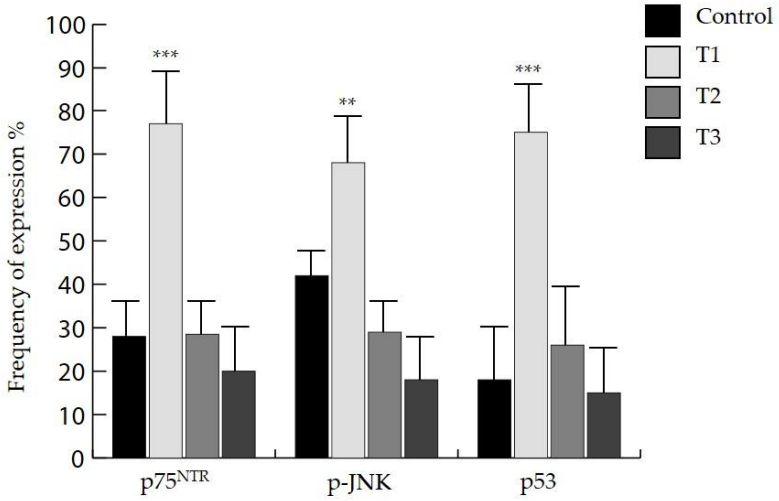


Figure 4. Frequency of expression of $p75^{NTR}$, p-JNK, and p53 in different tumor T stages versus control

2.5 Clinico-pathological significance of $p75^{NTR}$, p-JNK, and p53.

Interestingly, our analysis revealed that when compared to control tissue samples, all the proteins showed higher expression in samples with T1 tumor stage ($p < 0.001$) (Figure 4, Table 4).

Table 4. Association of NGF, TrkA, and $p75^{NTR}$ expression with clinico-pathological parameters

	nr	NGF		TrkA		$p75^{NTR}$	
		Positive (%)	*p*	Positive (%)	*p*	Positive (%)	*p*
T stage			0.91		0.40		0.001
T1	27	1 (0.03)		5 (5.9)		21 (77.7)	
T2	7	0 (0.0)		0 (0.0)		2 (28.5)	
T3	5	0 (0.0)		0 (0.0)		1 (20.0)	
T4	1	0 (0.0)		0 (0.0)		0 (0.0)	

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Vascular invasion		ns	ns		0.59
No	34	0 (0.0)	0 (0.0)	13 (38.2)	
Yes	6	0 (0.0)	0 (0.0)	3 (64.3)	
Histology		ns	ns		0.0001
Seminoma	26	0 (0.0)	0 (0.0)	25 (96.1)	
Non-seminomatous	14	0 (0.0)	0 (0.0)	4 (28.5)	

3. Discussion

As far as we are aware, this study represents the first description of the overexpression of $p75^{NTR}$ in TGCTs, particularly in testicular seminoma, and of its downstream signaling molecules, phospho-JNK and p53, whose expression decreases as tumor staging worsens. Different studies have proven that $p75^{NTR}$ plays opposite roles in the context of different cancers, as it acts as a tumor suppressor in carcinomas of prostate, bladder, stomach, and liver [18–20], whereas, in melanoma, pancreatic carcinoma, glioma, and breast cancer, $p75^{NTR}$ acts as a tumor-promoting function facilitating survival and invasion of cancer cells [21–23].

Although the involvement of $p75^{NTR}$ has been investigated in tumors affecting the female reproductive system, to our knowledge there are no studies that have explored the expression of $p75^{NTR}$ in TGCTs, in particular in testicular seminoma, which represents the most frequent testicular neoplasm in young men [1,24].

Previous studies have been carried out to identify new molecular markers for TGCTs. Preliminary reports demonstrated that most cells in seminoma express Pituitary-tumor-transforming-gene 1 (PTTG1) as well as Octamer-binding transcription factor 4 (OCT-4) and Krüppel-like factor 4 (KLF-4) [25]. The authors firstly demonstrated that PTTG1 marks some specific OCT4- and KLF4-positive tumor cells, mainly localized at the periphery of the neoplasm. In the intertubular infiltration areas, nests of cells expressing both OCT4/KLF4 and PTTG1 presence were consistent for a sub-population of tumor stem cells OCT4- and KLF4- positive [26]. In the present study, we aimed to investigate a new molecular pathway and found that the overexpression of $p75^{NTR}$ may have a pathogenetic role in testicular germ cell cancer development, which may occur in an NGF-independent manner [27,28], as confirmed by immunohistochemical Allred-score of negative NGF staining and its pro-survival receptor, TrKA, in tumoral tissue. Interestingly, Micera et al reported that the biological effect mediated by the NGF axis in both normal and cancer cells is related to the TrkA/$p75^{NTR}$ ratio and that the $p75^{NTR}$ expression might facilitate cell proliferation in the absence of TrkA [29].

We can speculate that $p75^{NTR}$ may be a biomarker of the transformed primordial germ cells, which represent the characteristic cellular pattern of testicular seminoma, in particular its pure forms [30]. The pathogenesis of overall TGTCs remains unexplored, although it is known that they originate from transformed gonocytes or undifferentiated spermatogonia [31]. Different observations using several malignancies refer to cells with a remarkable self-renewal potential and extensive proliferation capacity [32–34], expressing markers that characterize the stem cells of the original normal tissue [35] and that are strongly involved in growth and tumor propagation. A growing body of evidence has identified $p75^{NTR}$ as a robust cell surface biomarker for neural cancer-initiating or stem-like cells [36]. Interestingly, Okumura et al. demonstrated that in the stem/progenitor cell fraction of normal esophageal epithelial cells, $p75^{NTR}$ is necessary for tumor survival and maintenance [37]. Moreover, the *in vivo* study of Boiko et al. reported that melanoma tumor stem cells $p75^{NTR}$-positive, but not $p75^{NTR}$-negative, were remarkably capable of generating tumors, promoting metastasis, and maintaining self-renewal [38].

Furthermore, as expected, we observed that control testicular tissue expressed both total and phospho-JNK; and it has been reported that JNK is involved in regulating various testicular functions, like germ cell development and acrosome reactions, by controlling the expression of genes involved in the apoptosis or survival signaling pathways [39,40]. Concomitantly, we found that the overexpression of $p75^{NTR}$ in tumor tissue was

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accompanied by an increased nuclear expression of its downstream signaling, phospho-JNK. This result requires further investigation, as it has been extensively demonstrated that both $p75^{NTR}$ and JNK pathway activation can promote different biological effects, depending on cancer type [41]. Expression of $p75^{NTR}$ has been associated with apoptosis, through activation of JNK, which controls the expression of p53 tumor suppressor, and with cell survival, through the activation of nuclear factor-kB and AKT [42]. In addition to $p75^{NTR}$ and phospho-JNK overexpression, our analysis revealed a strong nuclear immunoreactivity of p53 in the tumor sections compared to control, highlighting that hyperactivation of $p75^{NTR}$ signaling in seminoma cells may promote cancer cell apoptosis. Furthermore, we hypothesized that $p75^{NTR}$ overexpression may represent a positive prognostic factor in seminoma, as we observed that $p75^{NTR}$, phospho-JNK, and p53 expression frequency decreases with staging progression, suggesting that $p75^{NTR}$ could represent a marker of tumor differentiation. These results are in agreement with previous reports, demonstrating an inverse association of $p75^{NTR}$ expression with the neoplastic progression of prostate cancer [43,44].

Our study has some limitations. First of all, this is a retrospective study based on an archive of collected samples coming from testicular neoplasms, therefore, the lack of access to patients' clinical information limited the evaluation of any relationship between immunohistochemistry findings and tumor aggressiveness. Besides, we used the area under ROC curve to assess the cutoffs for $p75^{NTR}$ positivity and to investigate the definition of scores better associated with clinical events. Although it may vary depending on tumor type, we used this approach to estimate the possible clinical significance of $p75^{NTR}$ as a biomarker. TMA method demonstrated accuracy for all proteins analyzed. The tumor heterogeneity, quite common in TGCTs, could be a limitation of this method. However, in this study, scores in triplicate for each histological subtype were used, in the attempt to minimize this limitation. Lastly, the present study was not aimed to clarify any molecular pathway that could yield to any relevant functional mechanism(s) linked to tumor progression. Further studies are warranted.

4. Materials and Methods

4.1 Antibodies

The following primary antibodies were used: anti- $p75^{NTR}$, anti-NGF, anti-TrkA, anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-JNK (phospho-JNK and total-JNK; Cell Signaling Technology, Milan, Italy).

Biotinylated goat-anti-rabbit, anti-mouse, anti-donkey IgGs were used as secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

4.2 Human tissues

All formalin-fixed and paraffin-embedded samples of TGCTs, stored from the 1st January 2015 to 31st December 2019 in the archives of the Division of Pathology, Hospital "A. Pugliese", Catanzaro (Italy), were collected. Only samples from primary tumors before chemotherapy were selected. The clinicopathological data collected included age, date of diagnosis, histological type, tumor grade (when applicable), and the presence of vascular invasion.

At the time of orchidectomy, all patients gave their informed consent to use the remaining portions of tissue specimens for research purpose after their primary use for routine histologic staining. Therefore, for this study, no formal ethical approval was required for processing archival testicular tissue.

4.3 Tissue Microarray (TMA) construction and validation

All samples were analyzed, independently, by two pathological experts to confirm the diagnosis and delimitation of tumor areas for TMA cores. For each sample, both the tumor area and the corresponding normal tissues were selected (when available and sufficient) for triplicate cores of 1.0 mm. For TMA validation, 10 samples were randomly selected. For

these 10 samples, the immunohistochemical analysis was performed on both TMA and whole sections, and the results were compared.

4.4 Histopathological analysis

Morphological studies were carried out by Haematoxylin Eosin staining.

4.5 Immunohistochemistry

The immunohistochemical experiments were carried out on paraffin-embedded sections from all samples. Sections of 5 μm thick, after heat-mediated antigen retrieval, were obtained. Immunodetection was performed at 4 °C overnight, using the specific primary antibodies anti-NGF (1:100), anti-TrKA (1:100), anti- $p75^{NTR}$ (1:100), anti-p53(1:100), anti-JNK (1:100), and anti-posho-JNK (1:100). Then, biotinylated IgG (1:600) was applied for 1 hour at room temperature, followed by avidin-biotin complex (ABC)/horseradish peroxidase (HRP). Immunoreactivity was visualized by using diaminobenzidine chromogen (DAB). Sections were also counterstained with hematoxylin. The specificity of the Abs was verified by using normal rabbit serum and normal mouse serum, respectively, instead of the primary Abs. Immunostained slides of tumor samples were visualized using an Olympus BX41 microscope and the images were taken with CSV1.14 software, using a CAM XC-30 for image acquisition.

4.6 Scoring system

Immunoreactivity for human neoplastic tissues was scored using the "Allred Score" [45], which combines a proportion and an intensity score. A proportion score was assigned representing the estimated proportion of positively stained tumor cells on a scale from 0 to 5. An intensity score was assigned by the average estimated intensity of staining in positive cells on a scale from 0 to 3. Proportion score and intensity score were added to obtain a total score that ranged from 0 to 8. A minimum of 100 cells were evaluated in each slide. Six serial sections were scored for each sample.

4.7 Statistical Analysis

The results obtained with the human samples were analyzed using Prism GraphPad (version 9.0). ROC curve was used to define the final score cutoff for positivity, based on the area under the curve. The frequency of protein expression and comparison with clinicopathological data as well as the differences in the scores between seminoma and control samples were analyzed using the one-way ANOVA. The Wilcoxon test was used after ANOVA as post-hoc test. The agreement between TMA and whole sections was evaluated by the accuracy of the method.

5. Conclusions

In conclusion, our results suggest that $p75^{NTR}$ may exert a pathogenetic role in TGCTs and that the loss of its expression may represent a marker of worse tumoral differentiation. However, the mechanism(s) underlying the controversial and paradoxical functions of p75NTR in different cancer cells are not entirely explained. Further studies are needed to investigate the relevance of our findings, to establish whether $p75^{NTR}$ may be considered a new marker of differentiated TGCT and a potential pharmacological target for the treatment in selected cases of TGCTs.

Author Contributions: Conceptualization AP and A.A.; Methodology: V.R., R.M., L.M., E.A.G. and S.L.V.; software D.L. and M.V.; validation L.T. and R.B., A.P., E.A.G. and S.L.V; writing—original draft preparation, A.P and V.R.; review and editing, E.A.G., E.C., A.B. and A.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding

Conflicts of Interest: The authors declare no conflict of interest.

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