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CXCR4 mRNA overexpression in high grade prostate tumors: Lack of association with *TMPRSS2-ERG* rearrangement

Silvia de Muga[a,e,1], Silvia Hernández[b,1,*], Marta Salido[c], Marta Lorenzo[a], Laia Agell[a], Núria Juanpere[a], José A. Lorente[d,f], Sergio Serrano[a,f] and Josep Lloreta[a,b]
[a]*Department of Pathology, Hospital del Mar-Parc de Salut Mar-IMIM, Barcelona, Spain*
[b]*Department of Health and Experimental Sciences, Universitat Pompeu Fabra, Barcelona, Spain*
[c]*Molecular Cytogenetics Laboratory, Department of Pathology, Hospital del Mar-Parc de Salut Mar-IMIM, Barcelona, Spain*
[d]*Department of Urology, Hospital del Mar-Parc de Salut Mar-IMIM, Barcelona, Spain*
[e]*Department of Biochemistry and Molecular Biology, Autonomous University of Barcelona, Barcelona, Spain*
[f]*Department of Morphologic Sciences, Autonomous University of Barcelona, Barcelona, Spain*

Abstract. The *TMPRSS2-ERG* fusion has been reported in 42 to 78% of prostate tumors. More than 90% of ERG-overexpressing tumors harbor the fusion. The relationship between the *TMPRSS2-ERG* fusion and prognosis is controversial. Different studies have suggested an association between CXCR4 and ERG overexpression resulting from the *TMPRSS2-ERG* rearrangement. The aim of this study was to investigate the relationship between *CXCR4* expression, *TMPRSS2-ERG* fusion and Gleason grade in prostate cancer. *TMPRSS2-ERG* rearrangement was investigated by FISH ($n = 44$), ERG protein by IHC ($n = 84$), and *CXCR4* by quantitative RT-PCR ($n = 44$). *TMPRSS2-ERG* rearrangement and ERG protein expression were present in almost 50% of the cases, without statistical differences between the different Gleason score groups. There was a very high concordance between FISH and IHC techniques (Kappa Index = 0.954). Seventy percent of Gleason $\geqslant 8$ prostate tumors overexpressed *CXCR4* mRNA, and the difference in *CXCR4* expression with Gleason < 8 cases was statistically significant ($p = 0.009$). There was no association between ERG protein and *CXCR4* mRNA expression. In conclusion, our results reveal for the first time that *CXCR4* overexpression is associated with high Gleason score prostate tumors, but that it is independent of the *TMPRSS2-ERG* rearrangement.

Keywords: CXCR4, TMPRSS2, ERG, rearrangement, overexpression, prostate cancer

Abbreviations

ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1
ERG	ETS related gene
ETS	E twenty-six
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescence in situ hybridization
IHC	Immunohistochemistry
PCA3	Prostate Cancer Antigen 3
PSA	Prostate-specific antigen
TMPRSS2	Transmembrane protease, serine 2

1. Introduction

Tomlins et al. [1] reported for the first time recurrent gene fusions of the 5'UTR (untranslated region) of the androgen regulated gene *TMPRSS2* with *ETS* transcription factor family members in prostate cancer. Different studies have confirmed the occurrence

[1]The two first authors have contributed equally to this paper.
*Corresponding author: Silvia Hernández, Department of Health and Experimental Sciences, Universitat Pompeu Fabra, Passeig Maritim 25-29, 08003-Barcelona, Spain. Tel.: +34 93 248 30 31; Fax: +34 93 248 31 31; E-mail: silvia.hernandez@upf.edu.

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of *TMPRSS2-ERG* fusion in 42 to 78% of prostate tumors [2–12].

TMPRSS2 and *ERG* are two genes located within 3 Mb on chromosome 21 (21q22). In addition to *ERG* or *ETV1*, other less frequent fusion variants between the 5'UTR of *TMPRSS2* and other *ETS* family members, such as *ETV4* [13] and *ETV5* [14], have been identified. Moreover, also other less common 5' fusion partner genes have been found in prostate tumors [3,15–17].

More than 90% of prostate tumors overexpressing *ERG* harbor the fusion between *ERG* and *TMPRSS2* genes. On the other hand, *ETV1* expression is found in 6–16% of prostate tumors, and only 1% of it is related to the *TMPRSS2-ETV1* fusion [1,17].

The different studies performed until now show marked discrepancies about the association between the *TMPRSS2-ERG* fusion and prognostic factors [4,7,18, 19,21]. Attard et al. [18] report a significant association between *TMPRSS2-ERG* rearrangement and high serum PSA, advanced tumor stage, and high Gleason grade. Other reports support an association with aggressive behavior but fail to relate it to Gleason grade [7, 22]. In contrast, some authors have found a statistical association with low Gleason grade, longer PSA progression-free survival and lower tumor stage [4,21].

In a similar way, some papers have indicated that ERG could have a proto-oncogenic role in prostate tumor initiation [2,23], whereas others have reported that aberrant ERG expression is a progression event in prostate tumorigenesis, and that *TMPRSS2-ERG* rearrangement does not suffice to initiate prostatic neoplasia [24].

Recently, Park et al. [25] developed a novel anti-ERG antibody, expression of which has a good correlation with the detection of *ERG* rearrangements by FISH. The protein product of TMPRSS2-ERG isoform, similar to almost all ETS fusions, is a truncated rather than a chimeric ERG protein and therefore it may result in an increased functional activity [26]. Thus, the functional consequences of the *TMPRSS2-ERG* fusion product are dependent on the downstream effects of ERG overexpression. Cai et al. [27] identified several consensus ERG transcription factor binding sites in the promoter region of *CXCR4*. In addition, *CXCR4*, and also *ADAMTS1*, have been found upregulated in the presence of ERG overexpression in prostate cancer, indicating that these two genes could be *ERG* downstream pathway genes [24]. CXCR4 is a chemokine receptor factor that belongs to the family of G protein-coupled receptors and has been shown to play a crucial role in a number of biological processes [28]. Several studies have suggested that CXCR4 overexpression could play an important role in prostate cancer metastases [29–35]. In prostate cancer cell lines, CXCR4 expression is regulated by the *TMPRSS2-ERG* fusion [24,27,32]. In *TMPRSS2-ERG* positive VPCa cells line, ERG and CXCR4 are highly expressed, and ERG binds to the *CXCR4* promoter sequences [27]. Prostate specific ERG expression has been associated with transcriptional upregulation of *CXCR4* [24]. There are no studies dealing with the relationship between *TMPRSS2-ERG* fusion and CXCR4 overexpression in clinical samples of patients with prostate cancer.

In this paper, we have analyzed the relationship between quantitative mRNA expression of *CXCR4*, *TMPRSS2-ERG* rearrangement assessed by ERG protein expression, and Gleason grade in a series of primary prostate tumors. In addition we have investigated the prevalence of the *TMPRSS2-ERG* rearrangement by FISH and by ERG IHC, and also their relationship with tumor grade.

2. Materials and methods

2.1. Patients and tumor samples

Seventy-five prostate tumors were selected retrospectively from the files of the Parc de Salut MAR Biobank (MARBiobanc) and 12 from the Tumor Bank of the Hospital Clínic-Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain. From these, 83 were needle biopsy or radical prostatectomy specimens and 4 were tumors found at autopsy, so-called latent tumors. All tumors were staged with the TNM classification. According to the current Gleason grading criteria, the prostate tumors were combined Gleason score = 6 ($n = 37$), combined Gleason score = 7 ($n = 32$) and combined Gleason score $\geqslant 8$ ($n = 18$). In 43 tumors we had only formalin-fixed paraffin-embedded (FFPE) tissues, in 3 tumors we had only frozen tissues and in 41 we had both types of samples.

The biopsy samples were collected between 2000 and 2011, with a follow-up time from 1 to 143 months (mean = 40.7). The autopsy samples dated from 1993 to 2009.

2.2. Fluorescence in situ hybridization (FISH) analysis of TMPRSS2-ETS fusion

To assess the genetic status of *TMPRSS2* and *ERG* genes, FISH was carried out on FFPE tissues from 44

prostate tumors. *TMPRSS2-ERG* gene rearrangement status was evaluated using break-apart probes. The following bacterial artificial chromosome (BAC) clones were used: RP11-35C4 5' and CTD-210307 3' for *TMPRSS2* gene; and CTD-2102A22 5' and CTD-2341018 3' for *ERG* gene. We used consecutive sections for hematoxylin-eosin (H&E) and FISH in each of the samples. FISH technique and analysis were performed as previously described [36].

A normal pattern, without rearrangement, was considered when the sample showed 2 pairs of juxtaposed red and green signals; a translocation pattern through deletion was identified when a juxtaposed red and green signal (yellow) and a single red or single green signal were observed and finally, a translocation pattern but without deletion was recognized when a juxtaposed red and green signal (yellow) and a single red plus a single green for the rearranged allele were seen.

2.3. *Evaluation of ERG protein expression by immunohistochemistry (IHC)*

Immunohistochemical analysis was carried out on FFPE tumor tissue in 84 out of the 87 prostate tumors using a primary rabbit anti-ERG monoclonal antibody (clone EPR3864, Epitomics, Burlingame, CA, USA) and the Dako Envision+System-HRP (DAKO, Glostrup, Denmark).

There were no significant differences in the intensity of ERG staining, so only two patterns of expression were considered: negative cases (without any detectable staining) or positive cases (with positive nuclear staining). We used endothelial cells as internal positive control in each slide (Fig. 1) [25].

2.4. *Total RNA extraction and CXCR4 quantitative real-time RT-PCR (qRT-PCR) analysis*

Total RNA was extracted from 44 frozen prostate tumor samples and from 4 non-tumor prostate samples, used as controls, as previously reported [37]. All samples contained at least 70% of tumor cells. Total RNA purity and quality were assessed with the NanoDrop® ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA.) and the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). According to combined Gleason score, tumors were: Gleason 6 ($n = 19$), Gleason 7 ($n = 16$) and Gleason $\geqslant 8$ ($n = 9$).

CXCR4 mRNA expression was analyzed by quantitative Real-Time PCR in the 44 samples, with the ABI PRISM 7500 Sequence Detection System and using the TaqMan® Gene Expression Assay probe and primer mix (Applied Biosystems, Foster City, CA, USA). The Assay Identification number for *CXCR4* was Hs00976734_m1, and the *GADPH* (4310884E) gene was used as internal control. The samples were run in triplicate and the mean value was calculated for each case. Non-neoplastic prostate samples (4 cases/ run) were used to normalize the data using the $\Delta\Delta$Ct method. Values > 2 (expression ratio in tumors vs non-neoplastic samples) were considered to indicate *CXCR4* mRNA overexpression.

2.5. *Statistical analysis*

Categorical variables are presented as frequencies and percentages. Fisher's Exact test was used to compare categorical variables among groups. A p-value less than 0.05 was considered statistically significant. Statistical analysis was performed using the SPSS statistical package version 15.0 (SPSS Inc., Chicago, IL, USA). The agreement between FISH and IHC techniques was calculated using the Cohen's Kappa Index.

3. Results

3.1. *Detection of TMPRSS2-ERG rearrangements by FISH and distribution according to combined Gleason score*

We have investigated by FISH the presence of the *TMPRSS2-ERG* rearrangement in a set of 44 tumors (Table 1). The *TMPRSS2* rearrangement was found in 19 (47.5%) of them, 12 through the deletion of one allele (most frequently 3') and 6 through translocation, while one case harbored both rearrangement and amplification of *TMPRSS2*. To assess whether the 3' partner of the fusion was the *ERG* gene, we have analyzed *ERG* status. The FISH test for *ERG* was successful in 37 cases and from these, *ERG* rearrangement was found in 15 (40.5%). The *TMPRSS2-ERG* fusion was confirmed in 14 of the 15 cases. In another case in which we found rearrangement of the *ERG* gene we could not perform *TMPRSS2* FISH analysis, thus we were not able to confirm *TMPRSS2* as the 5' partner of the fusion in this case. Also, in 4 *TMPRSS2* rearranged cases it was not possible to perform the FISH analysis for *ERG*, and in one *TMPRSS2* rearranged case, *ERG* was not rearranged but had a gain.

Finally, we found *TMPRSS2* and/or *ERG* gains but not rearrangement in 4 cases: in 2 cases we detected

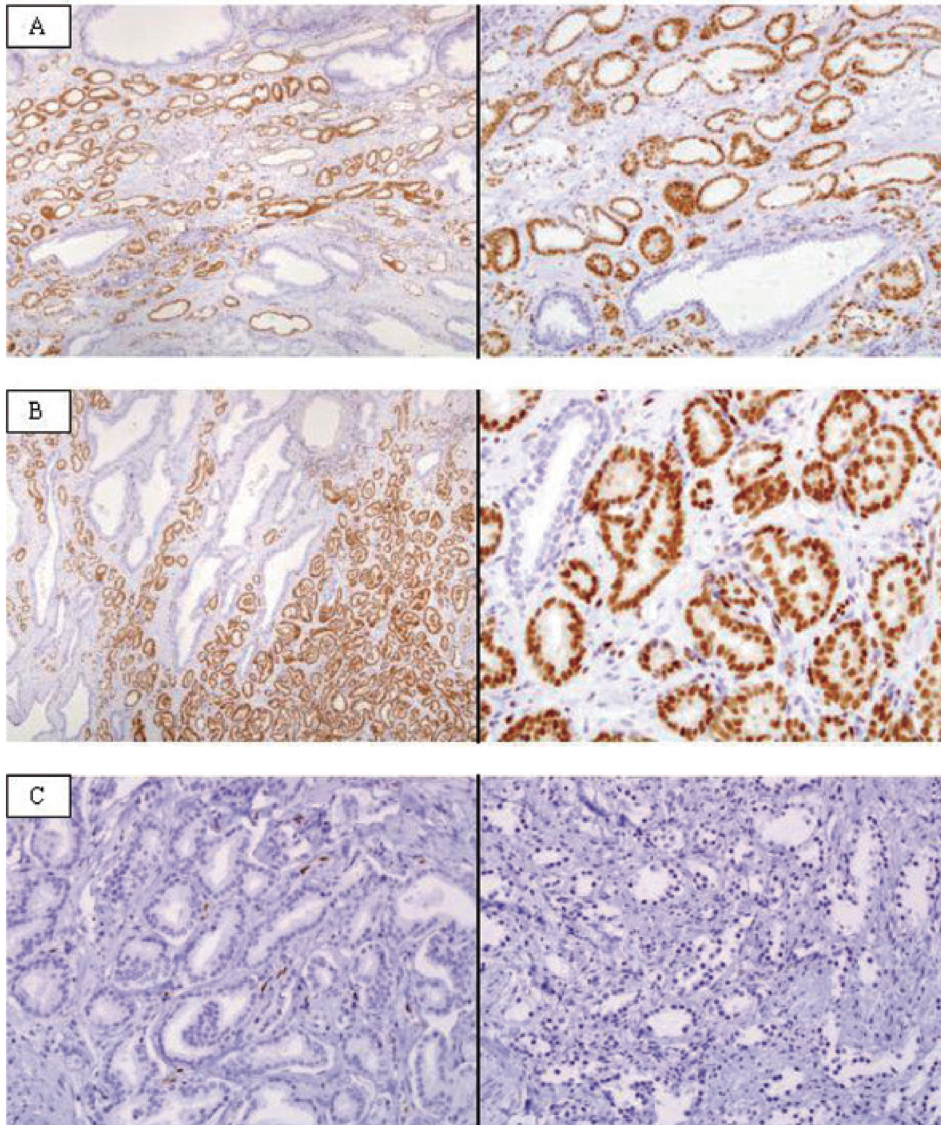

Fig. 1. ERG immunostaining (A–C). (A) Strong ERG expression in tumor cells of Gleason 6 prostate adenocarcinoma (x40 and x100). (B) Strong ERG expression in tumor cells of Gleason 7 prostate adenocarcinoma (x40 and x200). (C) Completely negative immunostaining in a Gleason 6 prostate adenocarcinoma (left) and in a Gleason 6 autopsy tumor (right) (x200). (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/CBM-2012-00288)

only a gain of the *TMPRSS2* gene and other two cases harbored both a *TMPRSS2* and an *ERG* gain.

According to combined Gleason score and considering only the 33 cases with information available for both genes, we found that 58.3% (7 of 12) of Gleason 6 group, 38.5% (5 of 13) of Gleason 7 and 37.5% (3 of 8) of the cases with Gleason score $\geqslant$ 8 harbored the *TMPRSS2-ERG* rearrangement. There were no statistically significant differences among the different Gleason categories.

3.2. ERG immunostaining analysis, distribution according to Gleason score and correlation with TMPRSS2-ERG rearrangement

As *TMPRSS2-ERG* fusion results in a marked nuclear ERG protein expression detected by IHC [3], we have analyzed ERG immunostaining in a set of 84 prostate tumors. Forty-four of them are the same in which we have previously checked the presence of rearrangement by FISH. As there is an excellent concordance between

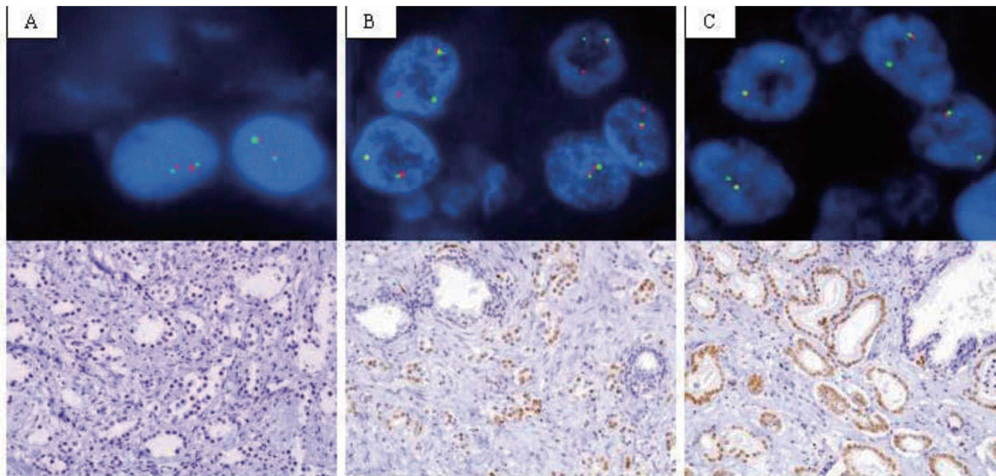

Fig. 2. *ERG* rearrangement by FISH and ERG protein expression by IHC. (A) Case without *ERG* rearrangement that did not express ERG protein. (B)(C) Cases with the *ERG* rearrangement detected by FISH, and expressing the ERG protein. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/CBM-2012-00288)

FISH and IHC techniques in the analysis of *ERG* gene, we decided not to do both techniques in all cases, and we assumed that ERG-positive cases had the rearrangement. Tumor nuclei were either positive or completely negative with the ERG antibody (Fig. 1). Forty-seven of 84 (56%) prostate tumors showed ERG protein expression. In two cases, there were ERG-positive and ERG-negative areas. The remaining cases showed homogeneous ERG expression. According to Gleason score, we found that 58.3% (21 of 36) of Gleason 6; 54.8% (17 of 31) of Gleason 7 and 53% (9 of 17) of Gleason $\geqslant 8$ cases showed ERG expression. This difference was not statistically significant.

From the 44 cases analyzed also by FISH, we found ERG expression in 21 cases (47.7%). Fourteen (66.7%) of the 21 cases with positive ERG immunostaining harbored the *TMPRSS2-ERG* rearrangement. In 5 cases, FISH analysis could not be completed for both genes and another case showed *TMPRSS2* but not *ERG* rearrangement. Finally, only one case without any rearrangement detectable by FISH, showed ERG protein expression. In these two cases with no detectable *ERG* rearrangement, ERG protein expression could be due to a type of alteration different from the *TMPRSS2-ERG* fusion. Interestingly, we identified also one case in which we found positivity for FISH as well as for IHC in a region of the tumor, while another region was negative for both techniques, confirming the good association between rearrangement and protein expression.

There is a statistical association between rearrangement and protein expression (Kappa Index = 0.954 (0.866–1.0; 95% CI)) (Fig. 2). We did not detect any *TMPRSS2-ERG* rearranged case with a negative ERG immunostaining.

Table 1
TMPRSS2 and *ERG* status detected by FISH analysis

Number of tumors	*TMPRSS2* status	*ERG* status
14	REARRANGED	REARRANGED
4	REARRANGED	Not successful
1	REARRANGED	GAIN
1	Not successful	REARRANGED
2	GAIN	GAIN
2	GAIN	WT[a]
14	WT	WT[a]
3	Not successful	WT[a]
3	WT	Not successful

[a]Wild type.

3.3. Quantitative CXCR4 mRNA expression and ERG protein expression

mRNA *CXCR4* expression was investigated by qRT-PCR in 44 prostate tumors for which we had adequate frozen samples available. From these, 19 tumors were Gleason 6, 15 were Gleason 7, and 10 were Gleason $\geqslant 8$. Based on the statistical analysis, the cut-off assumed for mRNA overexpression was > 2 and 14 of 44 (31.8%) prostate tumors showed *CXCR4* overexpression. According to the Gleason score, 26.3% (5 of 19) of Gleason 6 tumors, 13.3% (2 of 15) of Gleason 7 tumors and 70% (7 of 10) of the Gleason $\geqslant 8$ tumors overexpressed *CXCR4* (Fig. 3). Thus, *CXCR4* mRNA overexpression was statistically associated with high Gleason score (Fisher's Exact Test, $p = 0.009$). In 41 of these 44 samples, ERG immunostaining analy-

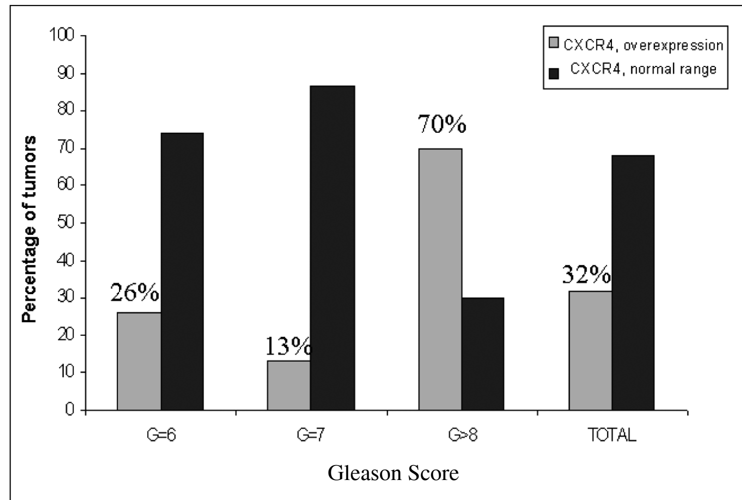

Fig. 3. *CXCR4* mRNA expression in prostate tumors according to the different Gleason grades.

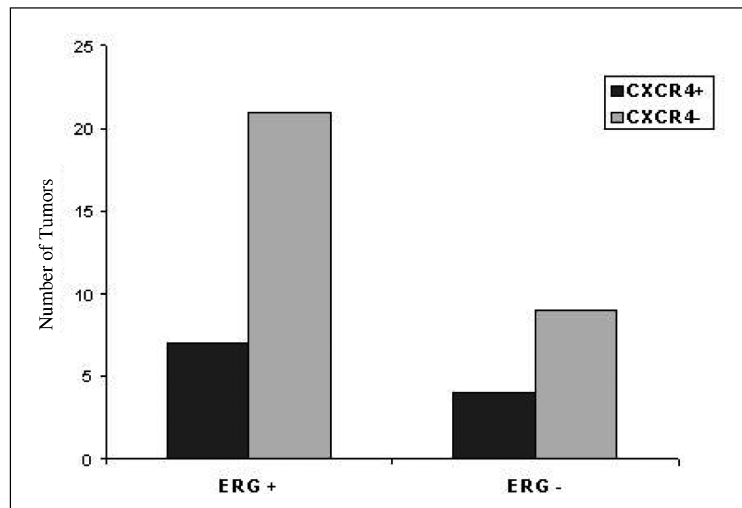

Fig. 4. Correlation between ERG protein expression and *CXCR4* mRNA overexpression.

sis was also performed and from these, mRNA overexpression was found in 11. Seven of these 11 cases (63.6%) showed positive expression of ERG protein. However, there was a higher percentage of cases (21 of 28, 75%) with ERG immunostaining without *CXCR4* mRNA overexpression (Fig. 4). Thus, there was no statistical association between ERG protein and *CXCR4* mRNA expression, indicating that *CXCR4* could be upregulated by an ERG independent mechanism.

4. Discussion

The presence of multiple types of rearrangement be-tween ETS family members and different 5' fusion partners is well documented in the literature, being the *TMPRSS2-ERG* the most frequently reported in prostate cancer and the most likely aberrant cause of ERG overexpression [1]. Moreover, the studies performed with multiple types of human neoplasms reveal that *TMPRSS2-ERG* rearrangement is a genomic alteration specific for prostate cancer and not found in other human neoplasms [38]. Recently, a urine assay for TMPRSS2-ERG plus urine PCA3 (Prostate Cancer Antigen 3) detection has been developed by Tomlins et al. [39], that seems to improve the stratification of patients with increased PSA and allows for a better identification of cases with significant cancer on biopsy.

There is controversy on the prognostic implications of the *TMPRSS2-ERG* fusion in prostate cancer and about the possibility that *TMPRSS2-ERG* rearrangement could be either an early or a late event in prostate carcinogenesis. In that sense, different studies have indicated that ERG could have a proto-oncogenic role in prostate tumor initiation [2,23]. Other studies report that the *TMPRSS2-ERG* fusion is not enough to initiate prostate neoplasia (nor PIN) and that other cooperating oncogenic lesions, such as *PTEN* losses, would be required [24–38,40].

Perner et al. [7] found a higher prevalence of *TMPRSS2-ERG* rearrangements in cases with high tumor stage and metastases, but without statistically significant differences. In the same series, cases with rearrangement through deletion showed a statistical trend for higher PSA biochemical recurrence rates when compared with prostate tumors without the fusion. Other reports support the association of *TMPRSS2-ERG* fusion with a more aggressive prostate cancer phenotype [18–20].

On the other hand, many studies have reported an association between rearrangement and low Gleason grade, and between *ERG* RNA expression (with or without rearrangement) and favorable prognostic factors [4,21].

In our series of primary prostate tumors, we have found a higher percentage of rearrangement in cases with Gleason $= 6$ compared to tumors with Gleason $\geqslant 7$, while the percentage of tumors with the rearrangement was very similar in Gleason $= 7$ and Gleason $\geqslant 8$ cases. Our results are in concordance with other reports that have found a larger proportion of FISH positive tumors in the low Gleason categories [18–20]. Nevertheless, similar to Demichelis et al. [19] we did not find any statistical association between fusion status and tumor grade.

A recent study, using a novel anti-ERG antibody, has shown a very good concordance between ERG protein immunostaining and *ERG* rearrangement detected by FISH in prostate cancer [25]. We have assessed the immunohistochemical expression of ERG to investigate the concordance between *TMPRSS2-ERG* rearrangement and ERG protein expression in our cases, as well as the association between ERG protein expression and the combined Gleason score of the tumors. Similar to previous reports [25,41], about half of the prostate tumors showed ERG protein expression. We also found a very good statistical concordance between FISH and IHC analysis, as in all cases with *TMPRSS2-ERG* rearrangement there was expression of the ERG protein. There were only two cases in which ERG expression did not correlate with *TMPRSS2-ERG* rearrangement. In these cases, ERG protein expression should be explained through another mechanism, different from the *TMPRSS2-ERG* fusion. ERG protein expression was limited only to the neoplastic glands, and most tumor samples were either positive or completely negative. In two tumors we detected heterogeneous expression, with a positive and a negative ERG region. In one of them, FISH analysis could be performed, and it revealed the *TMPRSS2-ERG* rearrangement in the ERG protein positive region, while the ERG negative region did not harbor the translocation. These findings further confirm the good association between rearrangement and ERG protein expression. According to the Gleason score, the percentage of ERG-positive tumors was similar in the Gleason 6 and 7 groups, but lower in the Gleason $\geqslant 8$ group. However, as in the *TMPRSS2-ERG* detection analysis, this difference was not statistically significant.

Cai et al. [27] identified several consensus ERG binding sites in the promoter region of *CXCR4* gene. *CXCR4* has been found up-regulated in prostate cancer cell lines in the presence of ERG overexpression, and TMPRSS2-ERG activation seems to be able to regulate *CXCR4* expression and subsequent metastatic spread [24]. Regardless of its possible relationship with ERG, CXCR4 by itself is a very interesting target to investigate in prostate cancer. The CXCR4-CXCL12 interaction has been involved in prostate cancer cell migration, invasion and bone metastasis [31,34,35]. *CXCR4* mRNA is overexpressed in prostate cancer cell lines as well as in a very high percentage of metastatic prostate samples [29,34,35]. Hart et al. [32] hypothesized that CXCR4 is the key of prostate metastatic implantation in bone narrow.

In order to assess the role of *CXCR4* in the different groups of primary prostate tumors and its relationship with the *TMPRSS2-ERG* rearrangement, we have analyzed the mRNA quantitative expression of *CXCR4*, along with the immunohistochemical detection of ERG expression. We have found *CXCR4* mRNA overexpression in about one third of the cases, with a statistically significant higher expression in Gleason $\geqslant 8$ prostate tumors. On the other hand, only a low percentage of tumors with Gleason $\leqslant 7$ in our series overexpressed this gene. Several studies have reported CXCR4 overexpression in both localized and metastatic prostate tumors by means of IHC [29,35,42,43]. Thus, Akashi et al. [29] showed that CXCR4 protein expression predicts poor prognosis in prostate cancer with bone metas-

tases. They did not find any statistically significant differences in CXCR4 expression with respect to pretreatment PSA level, combined Gleason score, clinical response to hormonal therapy, or extent of bone metastasis, but this may be due to the fact that their study was restricted to a subgroup of patients with advanced disease. On the other hand, Jung et al. [44] reported that local recurrence and distant metastases were associated with CXCR4 expression, but they did not find any association of this gene's overexpression with age, pretreatment PSA level, Gleason score, clinical stage nor biochemical recurrence.

To the best of our knowledge, only one previous study has investigated *CXCR4* mRNA expression in primary prostate cancer: Sun et al. [45], using secondary data analysis from a previous cDNA microarray study [45], reported increased *CXCR4* mRNA levels in prostatic hyperplasia (BPH), localized PCa and metastatic tumors, although no significant differences were found among BPH and the different tumor grade categories. In the present paper, we report for the first time that *CXCR4* mRNA is significantly overexpressed in a large percentage, over 70%, of prostate tumors with high combined Gleason score, but only in about 20.5% of tumors with Gleason scores 6–7. As we commented on previously, different studies have indicated that *TMPRSS2-ERG* rearrangement can lead to increased CXCR4 expression [24,27]. However, our results seem to indicate that there is no statistical association between ERG rearrangement and *CXCR4* quantitative mRNA expression. We hypothesize that only a low proportion of *CXCR4* overexpressing cases are due to the ERG rearrangement. Considering that the protein product of almost all ETS fusions, including the most common TMPRSS2-ERG isoform, is a truncated ETS protein rather than a chimeric protein [46], we can speculate that not all TMPRSS2-ERG fusion protein products will be able to activate ERG downstream targets such as *CXCR4* and *ADAMTS1* [27]. *TMPRSS2-ERG* rearrangement could represent only one of the mechanisms for CXCR4 aberrant overexpression, and high grade tumors could develop CXCR4 overexpression through alternative mechanisms. More studies will be required to assess the prognostic value of CXCR4 expression, and to investigate whether this molecule could help to identify a subset of low grade cases with *TMPRSS2-ERG* fusion that could be at a higher risk of progression.

In conclusion, our results support the lack of association between *TMPRSS2-ERG* fusion and histological grade in prostate cancer, as well as the excellent correlation between *ERG* rearrangement and ERG immunohistochemistry. On the other hand, our results also indicate that *CXCR4* mRNA expression is independent of the *TMPRSS2-ERG* rearrangement, and show for the first time a statistical association between the increased *CXCR4* mRNA and high grade prostate cancer. Further studies are needed to determine if CXCR4-overexpressing, low grade prostate tumors constitute a subset with a higher risk of progression.

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Conflict of interest

The authors declare no conflict of interest.

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