Copy number variations of \textit{E2F1}: a new genetic risk factor for testicular cancer

Maria Santa Rocca\textsuperscript{1}, Andrea Di Nisio\textsuperscript{1}, Arianna Marchiori\textsuperscript{1}, Marco Ghezzi\textsuperscript{1}, Giuseppe Opocher\textsuperscript{1,2}, Carlo Foresta\textsuperscript{1} and Alberto Ferlin\textsuperscript{1}

\textsuperscript{1}Department of Medicine, DIMED University of Padua, Padua, Italy  
\textsuperscript{2}Familial Tumor Unit, Veneto Institute of Oncology IOV-IRCCS, Padua, Italy

Abstract

Testicular germ cell tumor (TGCT) is one of the most heritable forms of cancer. In last years, many evidence suggested that constitutional genetic factors, mainly single nucleotide polymorphisms, can increase its risk. However, the possible contribution of copy number variations (CNVs) in TGCT susceptibility has not been substantially addressed. Indeed, an increasing number of studies have focused on the effect of CNVs on gene expression and on the role of these structural genetic variations as risk factors for different forms of cancer. E2F1 is a transcription factor that plays an important role in regulating cell growth, differentiation, apoptosis and response to DNA damage. Therefore, deficiency or overexpression of this protein might significantly influence fundamental biological processes involved in cancer development and progression, including TGCT. We analyzed \textit{E2F1} CNVs in 261 cases with TGCT and 165 controls. We found no CNVs in controls, but 17/261 (6.5\%) cases showed duplications in \textit{E2F1}. Blot analysis demonstrated higher E2F1 expression in testicular samples of TGCT cases with three copies of the gene. Furthermore, we observed higher phosphorylation of Akt and mTOR in samples with \textit{E2F1} duplication. Interestingly, normal, non-tumoral testicular tissue in patient with \textit{E2F1} duplication showed lower expression of E2F1 and lower AKT/mTOR phosphorylation with respect to adjacent tumor tissue. Furthermore, increased expression of \textit{E2F1} obtained \textit{in vitro} in NTERA-2 testicular cell line induced increased AKT/mTOR phosphorylation. This study suggests for the first time an involvement of \textit{E2F1} CNVs in TGCT susceptibility and supports previous preliminary data on the importance of AKT/mTOR signaling pathway in this cancer.

Introduction

Testicular germ cell tumors (TGCTs) are the most frequent solid malignancy in young men aged between 15 and 35 years. Most of TGCTs are sporadic, but the heritability is high, and positive family history represents a well-established risk factor (Nallu \textit{et al.} 2014, Rajpert-De Meyts \textit{et al.} 2016). Other known risk factors are a prior TGCT, infertility, cryptorchidism and testicular microlithiasis (Nallu \textit{et al.} 2014, Rajpert-De Meyts \textit{et al.} 2016). The genetic contribution to TGCT susceptibility is substantial, and over the last years, many studies have been focused on the role of genes potentially related to TGCT development (Kanetsky \textit{et al.} 2009, Ferlin \textit{et al.} 2010, Ferlin \textit{et al.} 2012).
Furthermore, genome wide association studies identified a number of single nucleotide polymorphisms (SNPs) associated with TGCT, suggesting a strong genetic component for this cancer (Rapley et al. 2009, Nallu et al. 2014, Litchfield et al. 2015, Rajpert-De Meyts et al. 2016). These new TGCT susceptibility loci contain biologically plausible genes encoding proteins important for male germ cell development, chromosomal segregation and the DNA damage response (Koster et al. 2014, Rajpert-De Meyts et al. 2016).

Recent evidence shows that copy number variations (CNVs) may have a biological impact on a variety of human diseases, including several forms of cancer (de Smith et al. 2008), and studies identified rare CNVs that might contribute to the oncogenesis of TGCT (Stadler et al. 2012, Edsgärd et al. 2013).

E2F1 (UniprotKB: Q01094) is a member of the E2F family, acting as a transcription factor with high binding affinity for the retinoblastoma tumor suppressor (RB) protein (Sengupta & Henry 2015). The interaction between E2F1 and pRB prevent E2F1-binding target genes containing the E2F-binding site and the transition from G1 phase of the cell cycle to S phase (Johnson 2000, Bertoli et al. 2013). Conversely, E2F1 liberation by binding with pRB, as it occurs in response to the loss of function of pRB, might affect cell cycle, increasing susceptibility to tumor development (Giacinti & Giordano 2006). No studies have been performed dealing with E2F1 and TGCT. Nevertheless, the possible role of the E2F1 pathway in male reproduction disorder is supported by the recent finding of Jorgez and coworkers who found altered CNVs or point mutation of E2F1 in approximately 7% of patients with non-obstructive azoospermia (NOA) (Jorgez et al. 2015).

In a previous in vitro study on a human hepatocellular carcinoma cell line, overexpression of E2F1 protein has been related to an altered cellular proliferation, triggering the signaling pathway that has a key player in mTOR, a serine-threonine kinases involved in biologically important cell processes (Ladu et al. 2008). Furthermore, a recent study focusing on mTOR as potential target for cancer treatment proposed that mTOR-activating mutations may be considered oncogenic and would contribute to TGCT development (Ichimura et al. 2016). In addition, Yaba and coworkers have recently shown a dislocation of mTOR and p-mTOR in human seminoma tissues, suggesting an involvement of these protein kinases in human testicular cancer (Yaba et al. 2016).

Taken together, these data suggest that E2F1 might be involved in male infertility; E2F1 is deregulated in many types of cancer; E2F1 might switch to Akt/mTOR pathway and mTOR pathway might be involved in TGCT carcinogenesis. Therefore, E2F1 is a good candidate for TGCT development.

In this study, we aimed to better understand whether an association between altered CNVs and expression of E2F1 and TGCT risk exists and, in that case, whether the Akt/mTOR signaling pathway is deregulated.

Material and methods

Ethical approval

The study has been approved by the Ethics Committee of the University-Hospital of Padova, and each participant gave his written informed consent. The study has been conducted in accordance with the principles expressed in the Declaration of Helsinki.

Subjects

We selected 261 men (146 with seminoma and 115 with non-seminoma cancer), referred to the Unit of Andrology and Reproduction Medicine of the University of Padova for sperm cryopreservation after orchiectomy, and 165 healthy normozoospermic males without TGCT consecutively recruited among men who were referred to the Unit of Andrology and Reproductive Medicine of the University of Padova for semen analysis as controls. Standard semen analysis was performed according to World Health Organization protocol (WHO 2010) after 2–7 days of sexual abstinence.

Testicular samples for Western blotting experiments derived from one subject with seminoma and E2F1 duplication (at the time of orchiectomy) and from one obstructive azoospermic man with E2F1 CNV=2 (at the time of testicular sperm extraction for sperm cryopreservation).

Cell culture and reagents

Embryonic carcinoma NTERA-2 cl.D1 [NT2/D1] (ATCC CRL-1973) cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco), with penicillin (100 U/mL), streptomycin (100 U/mL) and supplemented with 10% fetal calf serum in a humidified incubator at 37°C with 10% CO₂.

Plasmid transfection

NTERA-2 cl.D1 cells were used for transfection. Cells were seeded into 60 mm plates (2 × 10⁵ cells/well) and transfected...
with 1 µg/well of wild-type human E2F1 cDNA (sc112675) (OriGene Technologies Inc.; Rockville, MD, USA) using TurboFectin (OriGene Technologies Inc.). NTERA-2 cells transfected with empty vector (pcDNA3.1; Thermo Fisher Scientific Inc) were used as negative control. After 48 h, cells were harvested by scraping in ice-cold PBS and the pellet was stored at −80°C until use.

**DNA extraction**

Genomic DNA was extracted from peripheral blood leucocytes and cell pellet from NTERA-2 using QIAamp DNA Blood Midi Kit, according to the manufacturer’s protocol (Qiagen Inc.). The quality of the DNA was examined on a NanoDrop spectrophotometer (Thermo, Wilmington, DE, USA). Each sample was diluted at a final concentration of 5 ng/µL.

**Copy number variation analysis**

Quantitative real-time polymerase chain reaction (PCR) was performed using the TaqMan Copy Number Assay for E2F1 (Hs00576444_cn) (Applied Biosystems).

TaqMan CNV reactions were performed in triplicate using the FAM-dye-labeled assay for E2F1 and VIC-dye-labeled RNase P assay as a reference gene as described elsewhere (Jorgez et al. 2015). Real-time data were collected by the StepOne Plus 2.1 software, and ABI CopyCaller 2.0 software (Thermo Fisher Scientific Inc) was used for data analysis. Samples were run at least twice in independent assays to confirm results.

**Western immunoblotting**

Proteins were extracted from cell pellet and testicular biopsies by use of Bioplex Cell Lysis Kit (BioRad). Total protein content was quantified using Bradford Protein Assay Kit (BioRad), and protein samples (30 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel and transferred to polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Milano, Italy). The membranes were blocked for one hour with TBS-Tween containing 5% BSA or 5% nonfat milk and incubated with E2F1 (1:200, AbCam), p-Akt Ser473 (1:1000, Cell Signaling Technology), Akt (at 1:1000, Cell Signaling Technology), p-mTOR Ser2448 (1:1000, Cell Signaling Technology), mTOR (1:1000, Cell Signaling Technology) and Beta-actin (1:5000, AbCam) primary antibodies overnight at 4°C. Secondary antibodies were peroxidase-conjugated goat anti-mouse antibody (1:5000, BioRad) and goat-anti-rabbit antibody (1:10000, Bio-Rad).

**Immunoreactive proteins were visualized using the enhanced chemiluminescence system detection**

**Table 1**  Basic characteristic of the subjects. Data are reported as mean ± s.d.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls (n = 165)</th>
<th>TGCT cases (n = 261)</th>
<th>P valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>36.85 ± 7.6</td>
<td>38.35 ± 7.11</td>
<td>0.08</td>
</tr>
<tr>
<td>History of cryptorchidism (n, %)</td>
<td>0</td>
<td>32 (12.2)</td>
<td>–</td>
</tr>
<tr>
<td>Family history of TGCT (n, %)</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>TGCT histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seminoma (n, %)</td>
<td>–</td>
<td>145 (55.6)</td>
<td>–</td>
</tr>
<tr>
<td>Non seminoma (n, %)</td>
<td>–</td>
<td>116 (44.4)</td>
<td>–</td>
</tr>
<tr>
<td>Semen characteristicsb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Semen volume (mL)</td>
<td>3.64 ± 1.51</td>
<td>3.32 ± 1.74</td>
<td>0.12</td>
</tr>
<tr>
<td>Sperm concentration (mill/ejaculate)</td>
<td>66.68 ± 46.90</td>
<td>27.85 ± 34.79</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sperm count (mill/mL)</td>
<td>206.5 ± 143.27</td>
<td>82.99 ± 110.06</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Forward motility (%)</td>
<td>53.53 ± 17.90</td>
<td>27.21 ± 22.50</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sperm normal morphology (%)</td>
<td>11.23 ± 7.75</td>
<td>12.06 ± 7.39</td>
<td>0.38</td>
</tr>
<tr>
<td>Reproductive hormonesc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone (nmol/L)</td>
<td>N.A.</td>
<td>15.16 ± 5.79</td>
<td>–</td>
</tr>
<tr>
<td>Estradiol (pmol/L)</td>
<td>N.A.</td>
<td>117.17 ± 231.30</td>
<td>–</td>
</tr>
<tr>
<td>FSH (IU/L)</td>
<td>N.A.</td>
<td>11.67 ± 12.25</td>
<td>–</td>
</tr>
<tr>
<td>LH (IU/L)</td>
<td>N.A.</td>
<td>6.31 ± 4.70</td>
<td>–</td>
</tr>
<tr>
<td>E2F1 duplications (CNV = 3) (n, %)d</td>
<td>0 (0%)</td>
<td>17 (6.5%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*P values for continuous variables were calculated using multivariate analysis of variance with Bonferroni correction for multiple comparisons. Bold indicates P<0.05. Standard semen analysis was performed according to World Health Organization protocol (WHO 2010). Serum levels of FSH, LH, estradiol and testosterone were evaluated by commercial electrochemiluminescence immunoassay methods (Elecsys 2010; Roche Diagnostics). χ² test was used to compare the two groups.

TGCT, testicular germ cell tumour; FSH, follicle-stimulating hormone; LH, luteinizing hormone; NA, data not available; CNV, copy number variations.
kit (ECL, PerkinElmer) and detection with ChemiDoc
XRS + System (Bio-Rad).

### Statistical analysis

Statistical analyses were performed using SPSS 20.0 for Windows (SPSS). Pearson’s chi-square test, or Fisher’s exact test when expected frequency was five or less, was used to examine the differences in CNVs occurrence between cases and controls and between the two testicular cancer types (seminoma and non-seminoma). *P* values <0.05 was considered as statistically significant.

### Results

Table 1 shows general subjects characteristics, age, semen parameters and reproductive hormones. Patients and controls did not differ for age, semen volume and sperm morphology, whereas in TGCT subjects, sperm concentration, sperm total count and sperm motility were lower compared to those in controls.

Analysis of *E2F1* CNV found a statistically significant number of TGCT cases with duplications (17/261, 6.5%) with respect to controls (0%, *P*<0.001, Table 1). In particular, the frequency of CNVs >2 was significantly higher (*P*= 0.04) in patients with non-seminoma subtype of TGCT compared with that in seminoma (Table 2). No difference was found in the frequency of CNVs >2 between subjects with normozoospermia and oligozoospermia (Table 2).

Western blot analysis for *E2F1* performed on testicular biopsies showed a correlation between copy number and expression, as increased protein levels of *E2F1* was found in samples with *E2F1* duplication (Fig. 1A and B). Furthermore, in testicular seminoma samples with *E2F1* CNV = 3, the expression of both p-mTOR and p-AKT was also increased compared with that in control (Fig. 1C and D).

| Table 2 Clinical characteristics of the 261 subjects affected by testicular cancer subdivided on the basis of TGCT subtype and sperm count. |
|---|---|---|
| **Histology** | Seminoma | Non seminoma |
| E2F1 CNV = 2 | 140 | 104 |
| E2F1 CNV = 3 | 5 | 12 |
| **Sperm count** | | |
| Normozoospermia | Azo-oligozoospermia |
| (≥39 × 10⁶ sperm/ejaculate) | (<39 × 10⁶ sperm/ejaculate) |
| E2F1 CNV = 2 | 130 | 114 |
| E2F1 CNV = 3 | 6 | 11 |

*Pearson’s chi-square test, or Fisher’s exact test when expected frequency was five or less, was used to examine the differences in CNVs frequencies between the two testicular cancer types (seminoma and non-seminoma) and between normozoospermic and azo-oligospermic cases.
Additionally, we examined the expression levels of E2F1 in adjacent non-tumor testicular tissue obtained at least 1 cm away from the clear edge of the tumor and compared with cancer testicular tissue. As shown in Figure 2, E2F1 expression and AKT/mTOR phosphorylation were higher in cancer testicular tissue compared with those in the adjacent normal testicular tissues.

Furthermore, to evaluate the effect of E2F1 on Akt/mTOR pathway, E2F1 plasmid was transfected into NTERA-2 cells, which constitutively carry two copies of E2F1, to mimic the condition occurring in subjects with three copies of the gene. In E2F1 plasmid-transfected cells, the expression of E2F1 and its downstream targets Akt/mTOR was upregulated with respect to control NTERA-2 cells (Fig. 3).

Discussion
This is the first study to show an association between E2F1 duplications and TGCT. Increased copy number of E2F1 is associated with higher E2F1 protein expression in testicular tumor samples and higher expression of phosphorylated form of Akt and mTOR, suggesting a E2F1-dependent deregulation of Akt/mTOR signaling pathway. The expression of the E2F1 protein and the phosphorylation of AKT/mTOR was higher exclusively in tumor tissue, compared with corresponding normal tissue of the same patient with duplication of E2F1, suggesting that E2F1 overexpression, and the consequent upregulation of its supposed downstream effectors (p-AKT/p-mTOR), depends exclusively on germline E2F1 duplication and are involved in tumorigenesis. Interestingly, we confirmed in vitro that overexpression of E2F1 in testicular cell line induced enhanced phosphorylation of Akt and mTOR, supporting the role of this pathway in testicular tumorigenesis.

This study therefore suggests for the first time an involvement of E2F1 CNVs in TGCT susceptibility and supports the involvement of E2F1 in different cancers and of Akt/mTOR signaling pathway in TGCT.

The E2F family of transcription factors controls the expression of genes involved in cell proliferation, differentiation and apoptosis (Huang et al. 1997, Meng & Ghosh 2014). Generally, E2F1 promotes the activation of p53-dependent or -independent cell death pathway, working as tumor suppressor. However, E2F1 can also behave as an oncogene inducing hyperproliferation and...
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hyperplasia, depending on environmental context. In particular, overexpression studies revealed the oncogenic activity of E2F1 (O’Connor & Lu 2000, Johnson & Degregori 2006), whereas loss-of-function studies revealed the tumor suppressor properties of E2F1 (Field et al. 1996, Conner et al. 2000).

Furthermore, studies on KO mice and transgenic mice overexpressing E2F1 showed that E2F1 may be an important transcriptional regulator for male reproduction (Yamasaki et al. 1996, Hoja et al. 2004). This hypothesis is supported by animal studies in which E2F1−/− male mice displayed testicular atrophy with severe loss of spermatogonia (Hoja et al. 2004) and mice overexpressing E2F1 showed severe atrophy of the seminiferous tubules due to increased apoptosis of germ cells (Agger et al. 2005).

Recently, Jorgez and coworkers focused on the role of E2F1 in human male fertility, and they identified CNVs and point mutations in approximately 7% of patients with non-obstructive azoospermia, suggesting a key role for this transcription factor in spermatogenesis and testis development (Jorgez et al. 2015). Another recent study further supports a fundamental role for E2F1 during spermatogenesis, mainly by regulating apoptosis during germ cell development (Rotgers et al. 2015).

Aberrant expression of E2F1 has been documented in a wide variety of human cancers and, in particular, overexpression of E2F1 has been related to advanced disease and poor prognosis (Zhang et al. 2000). Therefore, our study expands the form of cancers that may be associated with E2F1 deregulation. We have no clear hypothesis to explain the stronger association of E2F1 duplications with non-seminoma with respect to seminoma. However, it is known that non-seminomas generally grow and spread more quickly than seminomas. In general, we can speculate that anomalous expression of E2F1, induced by CNVs, might trigger the upregulation of E2F1 target genes and consequently the activation of signaling cascades related to cell proliferation, invasion and metastasis, in agreement with the hypothesis of Engelmann & Pützer on the oncogenic role of E2F1 (Engelmann & Pützer 2012).

The deregulation of Akt/mTOR pathway observed in association with E2F1 duplications is particularly interesting because this pathway has a key role in several cancers, including TGCT as evidenced recently (Wang et al. 2016). Interestingly, this pathway is also under consideration for chemotherapy treatment protocols of testicular cancer (Ichimura et al. 2016, Yaba et al. 2016).

In conclusion, we hypothesize that subjects with abnormal copy number of E2F1 gene are at increased risk of developing testicular cancer than subjects with normal copy number, and this risk is associated with deregulation of E2F1/AKT/mTOR pathway, suggesting for the first time an oncogenic role of E2F1 in the tests.

Moreover, further studies would be needed to clarify if E2F1 may contribute to human infertility considering the difference in percentage between normozoospermic and oligozoospermic men, although not statistically significant.

Therefore, this is the first demonstration that E2F1 may act essentially as an oncogene in TGCT and that E2F1 duplications are associated with this cancer. Further studies are necessary to confirm our findings in larger samples, including familial TGCT, bilateral forms of TGCT, TGCT cases associated with history of cryptorchidism and TGCT refractory to standard chemotherapy protocols.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M S R performed the analysis of CNVs, critically analyzed the data and drafted and revised the manuscript. A D N contributed to interpretation of data and to the writing of manuscript. A M acquired CNV data. M G performed clinical analysis of subjects. G O selected the subjects. C F conceived the project and revised manuscript critically. A F designed the work, selected the subjects, performed clinical analysis, supervised the experimental procedures and results and wrote the manuscript.

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