RESEARCH NOTE

SPANX-B and *SPANX-C* (Xq27 region) gene dosage analysis in Down's syndrome subjects with undescended testes

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Introduction

Down's syndrome (DS) is one of the most common numerical chromosomal aberrations, usually caused by trisomy of chromosome 21, and is frequently complicated with congenital heart defects, duodenal obstruction and other conditions including undescended testis (UDT) (Fonkalsrud 1970). The incidence of undescended testes in DS was reported to be 6.52% (Chew and Hutson 2004) while the incidence of UDT in the first year is approximately 0.2%–0.8% in the normal population (Benson *et al*. 1991; Ichiyanagi *et al*. 1998). Rapley *et al*. (2000) provided evidence for a testicular germ-cell tumours (TGCT) predisposition locus at Xq27; the authors obtained an hlod score of 4.7 from families with at least one bilateral case, corresponding to a genome-wide significance level of $P = 0.034$. The proportion of families with undescended testis linked to this locus was 74%. *SPANX* (sperm protein associated with the nucleus in the X chromosome) gene family maps in the same chromosomal region and seven highly homologous genes belonging to this family have been described (*SPANX-A1, SPANX-A2, SPANX-B1, SPANX-B2, SPANX-C, SPANX-D* and *SPANX-E*) according to the human genome database build 36.2. These genes, made up of two exons separated by a small intron of ≈ 650 bp, are expressed in sperm cells (Westbrook *et al*. 2000) and in many tumours (Wang *et al*. 2003; Zendman *et al*. 2003; Westbrook *et al*. 2004). Moreover, expression of *SPANX* genes has been demonstrated in TGCT (Salemi *et al*. 2006). The function of *SPANX* gene-encoded proteins is currently unknown, and it is also not known if all the members or some of them are normally expressed in the testis (Westbrook *et al*. 2000). Evidence suggests that *CTp11*, which is 100% homologous to *SPANX-C*, is expressed in tumours such as melanoma (Zendman *et al*. 2003), and *SPANX-B* in myeloma and other haematological malignancies (Wang *et al*. 2003; Zendman *et al*. 2003). *SPANX-C* mRNA was found expressed in normal tissues and in embryonal carcinomas of the testis (Salemi *et al*. 2006). Further, it is very difficult to design primers adequate for gene-specific PCR amplification within the SPANX locus. For this reason, we decided to focus our study on *SPANX-C* and *SPANX-B* genes. The aim of this study is to evaluate the genetic variability of *SPANX-B* and *SPANX-C* in D.UDT (Down's syndrom patients affected by undescended testis) compared with D (Down's syndrom patients without undescended testis) and Nm (normal population).

Materials and methods

Patients

A total of 110 subjects were enrolled for this study at the IRCCS Oasi Institute, Troina (Italy). They included: 20 male D.UDT (range 3–20 yr), 44 male D (range 2–25 yr) and 46 normal males (range 3–26 yr). The DS cases and controls were recruited after family and personal informed consent in a specialized centre receiving only patients from Sicily. The

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diagnosis of undescended testis has been found with inspection and palpation of the testis and the scrotum, and subsequently confirmed by ultrasound.

DNA extraction

DNA was isolated from a lymphocyte-enriched fraction of whole blood with NUCLEON BACC3 for extraction of genomic DNA kit (Amersham Pharmacia Biotech, Milan, Italy) using protocol modified after Anello *et al*. (2001).

Primers

Primers for multiple PCR were newly designed according to the manufacturer's recommendation with Oligo DNA/RNA primer analysis software version 4.1 (Rychlik and Rhoads 1989) (table 1). We designed a forward primer for *SPANX-B* genes on the first exon with a 5′FAM fluorophore corresponding to a unique 18-bp insertion present only in the *SPANX-B* genes, and a reverse primer on the second exon (forward: 5′-CCAATGAGGCCAACAAGA-3′; reverse: 5′-GGTCTTTCAGTCGTTAT-3′), with a product size of 879 bp.

For *SPANX-C*, we designed a forward primer on the first exon with a 5′HEX fluorophore and a reverse primer on the second exon. The sequence of the *SPANX-C* forward primer shares 100% homology with the corresponding sequence of the first exon of *SPANX-C* (forward: 5′-GTGAATCCAACGAGGTGAATG-3′; reverse: 5′-GAATTCCTCCTCCTCCATTTG-3′) with a product size of 862 bp.

For the *CDR1* control gene, the forward primer with a 5′HEX fluorophore was designed before the start codon and the reverse primer after the stop codon of the unique exon (forward: 5′-ACCTGGAGATATAGGAAGAC-3′; reverse: 5′-AAATACAGATCTTCCAGCTAA-3′) with a product size of 977 bp.

Semi-quantitative fluorescent multiplex PCR

The semi-quantitative fluorescent multiplex PCR dosage analysis was performed essentially as described by Rowland *et al*. (2001) and Salemi *et al*. (2008) to identify gene dosage classes (GDC) of the *SPANX-B* and *SPANX-C* genes. Two segments of *SPANX-B* and *SPANX-C* genes were co-amplified in a fluorescent multiplex PCR with the *CDR1* control gene. Multiplex PCR reactions were carried out in 50 μl reaction volumes containing 250 ng DNA, 10–15 pmol of each primer, 0.4 mM of each dNTP, 1 mM $MgCl_2$, 1× reaction buffer (Euroclone, Milan, Italy) and 5 U of EuroTaq DNA Polymerase (Euroclone, Milan, Italy). Thermocycling conditions were as follows: 95°C for 5 min, then 20 cycles of 95°C, 40 s; 60°C, 40 s; 72°C, 40 s; 20 PCR cycles were used to ensure the reaction was kept within the exponential phase. A final step of 10 min at 72°C was also included. To check for the presence of amplification, PCR samples were

Table 1. *SPANX-B* gene dosage classes (GDC).

	Nm	D	D.UDT			*P (df = 1)		
GDC	N°	N°	N°	RPA	SE	Nm vs D	Nm vs D.UDT	D vs D.UDT
1	9	5	5	0.17	0.013	0.4341	0.8660	0.3071
2	7	6	3	0.32	0.006	0.9310	0.7257	0.8085
3	7	6	2	0.50	0.013	0.9310	0.8592	1.0000
4	3	1	2	0.60	0.020	0.6411	0.9878	0.4730
5	2	7	3	0.72	0.042	0.1817	0.3188	0.7806
6	7	4	0	0.84	0.006	0.5720	0.1585	0.4034
7	3	2	1	1.13	0.014	0.9592	0.7460	0.5767
8	1	1	0	1.34	0.022	0.4943	0.6658	0.6835
9	2	3	1	1.64	0.050	0.9592	0.5989	0.7806
10	2	2	0	2.18	0.033	0.6411	0.8684	0.8684
11	1	2	1	2.50	0.011	0.9698	0.8684	0.5767
12	0	1	0	2.94	–	0.9822	–	0.6835
13	2	2	0	3.18	0.051	0.6411	0.8684	0.8684
14	0	1	0	3.38	–	0.9822	–	0.6835
15	0	1	0	3.74	–	0.9822	–	0.6835
16	0	0	2	5.94	0.009	–	0.8684	0.8684
Total	46	44	20					

GDC, gene dosage classes; Nm, normal population; D, Down's syndrome patients without undescended testis; D.UDT, Down syndrome patients affected by undescended testis; RPA, ratio peak area of *SPANX-B* gene/peak area of *CDR1* gene; SE, standard error; *, chi-square test with the Yates' correction; df, degrees of freedom.

analysed on 2% agarose gel and observed using a UV transilluminator.

Capillary electrophoresis

Two μl of the PCR products were mixed with 12 μl of formamide and 1 μl of GeneScan 2500 ROX size standard (Applied Biosystems, Foster City, USA). The mixture was denaturated at 96°C for 3 min and placed on ice until further analysis. Capillary electrophoresis was performed using POP7 gel and ABI PRISM 3130 Genetic Analyser (Applied Biosystems, Foster City, USA). Fragment sizes and peak areas were determined by GeneScan Analysis 3.1.2 software (Applied Biosystems, Foster City, USA).

Data processing and statistical analysis

The data area of each amplified gene was normalized as described in Koolen *et al*. (2004) and Salemi *et al*. (2008). For each individual, we usually define relative probe signals, dividing each measured peak area of *SPANX-B* and *SPANX-C* by the *CDR1* peak area of that sample. The ratio of each individual probe relative area is then normalized to that obtained from all DNA case and control samples. GDC were determined for both *SPANX-B* and *SPANX-C* genes. For each identified class, the standard error of the mean of normalized areas (SE) was calculated with the following formula: SE = s/(square root of n) where s is the standard deviation and n is the number of observations. The chi-square test with Yates' correction was used to compare the frequencies of each class found in normal individuals and DS patients with or without undescended testis. A statistically significant difference was accepted when the P value was lower than 0.05.

Results and discussion

The analysis of *SPANX-B* gene in control samples revealed the presence of 12 GDC, 15 classes were detected in D, and nine GDC in D.UDT (table 1). Statistical analysis showed nonsignificant differences between the three populations (D, D.UDT and controls, respectively) (table 1).

The analysis of *SPANX-C* in control samples and in D revealed the presence of 10 and 12 GDC, respectively. Finally, eight classes in D.UDTS were observed (table 2). A statistical significant difference for the class B (χ^2 = 4.04; P = 0.0445; table 2) was found comparing D.UDT and controls. In addition, the comparison between D and controls showed a statistical significance for the class F (χ^2 = 6.23; P = 0.0125; table 2).

This study confirms the presence of the genetic variability for *SPANX-B* and *SPANX-C* genes in the normal population and in the DS patients. Such kind of semi-quantitative analysis is not able to reveal the copy number of genes; however, the number of *SPANX-B* GDC identified (n = 16) is close to the *SPANX-B* copy number polymorphism reported by Kouprina *et al*. (2005).

Similarly, our *SPANX-C* classes might represent copy number variation of this gene in the general population, a result so far undetected because of the difficulty of constructing primers/probes specific for *SPANX-C* because of the

Table 2. *SPANX-C* gene dosage classes (GDC).

	Nm	D	D.UDT			*P (df = 1)		
GDC	N°	N°	N°	RPA	SE	Nm vs D	Nm vs D.UDT	D vs D.UDT
A	11	10	7	0.00	–	0.9074	0.5295	0.4684
B	1	7	4	0.18	0.004	0.0551	**0.0445**	0.9644
C	2	8	2	0.51	0.005	0.0798	0.7476	0.6425
D	4	8	3	0.67	0.011	0.3110	0.7202	0.9644
E	2	4	1	0.97	0.024	0.6319	0.5989	0.9499
F	10	1	1	1.15	0.015	**0.0125**	0.1876	0.8464
G	0	1	0	1.42	–	0.9910	–	0.6835
H	6	1	0	1.56	0.020	0.1302	0.2194	0.6835
I	7	1	0	1.79	0.014	0.0740	0.1585	0.6835
L	1	1	0	1.92	0.022	0.4943	0.6658	0.6835
M	0	1	0	2.06	–	0.9822	–	0.6835
N	0	0	1	2.34	–	–	0.6658	0.6835
O	2	1	1	2.63	0.028	0.9698	0.5989	0.8464
Total	46	44	20					

GDC, gene dosage classes; Nm, normal population; D, Down's syndrome patients without undescended testis; D.UDT, Down syndrome patients affected by undescended testis; RPA, ratio peak area of *SPANX-C* gene/peak area of *CDR1* gene; SE, standard error; *, chi-square test with the Yates' correction; df, degrees of freedom and the bold face indicates the statistical significant results with $P < 0.05$.

high sequence similarity between *SPANX-A/D* genes. According to database build 36.2, the forward primer that we have used has a 100% identity with *SPANX-C* only. The lack of amplification products for *SPANX-C* in class A adds further evidence of primer specificity; however, the lack of amplification in class A was not necessarily due to absence of the gene. In fact, the presence of SNPs has been already reported in the *SPANX-C* sequence where we built the forward primer (Kouprina *et al*. 2005), or alternatively novel gene conversion/depletion events may have occurred, caused by segmental duplications (SDs) and/or rearrangements. The Xq27 region is characterized by a high density of SDs (Sharp *et al*. 2005). Because of the different orientation of SDs, their recombinational interactions may result in deletions, duplications and inversions of the *SPANX*-containing genomic region. The differences in gene dosage of *SPANX-C* class B between D.UDT group and normal subjects and of *SPANX-C* class F between D group and normal controls could be due to these mechanisms.

In this study (data not shown), the PCR reactions were also performed using only *SPANX-C* primer pairs, and in all cases of class A the absence of amplification was confirmed; further, D.UDT samples and 11 random chosen male normal samples were analysed for single PCR amplification of *SPANX-C, SPANX-B* and *CDR1* gene. Single PCR analysis confirmed the results of multiplex PCR. Sequencing of the Xq27 region in DS subjects with or without undescended testes belonging to different gene dosage classes has been previously performed to exclude large scale deletion and insertion (Salemi *et al*. 2008). In particular, the polyalanine tract of the *sox-3* and part of the unique exon of the *LDOC1* gene have been analysed. No deletions or sequence variations have been reported (Salemi *et al*. 2008).

Some of the control subjects used in this study already been used in a previous study (Salemi *et al*. 2007). In these subjects, sequencing of the *sox-3* gene has been performed and no sequence variations or deletions have been reported.

In the study of Salemi *et al*. (2008), 17 subjects with melanoma and 99 normal subjects were studied with semiquantitative fluorescent multiplex PCR dosage analysis to identify GDC of the *SPANX-B* and *SPANX-C* genes. In this work they obtained gene classes that overlap with ours.

From our point of view, the most relevant finding of this study is the statistically significant difference for class B of the *SPANX-C* gene in D.UDT (class B: $P = 0.0445$; table 2) and for class F in D (class F: $P = 0.0125$; table 2), as compared with normal subjects, respectively.

In fact, the series of controls in our study and in our previous studies suggests that the possible presence of a genotype (corresponding to class B) specifically associated with DS patients with undescended testis and a genotype (corresponding to the class F) underrepresented in the DS patients sample analysed.

Further studies will be necessary to evaluate these conclusions since the Xq27 region undergoes wide genomic rearrangements that cause deletions, duplication and/or segmental inversions, associated with undescended testes and other pathologies. Additional studies should also be conducted in normal subjects with undescended testis to evaluate the possible implication of other genes mapping in the Xq27 region.

References

Anello G., Guéant J., Romano C., Barone C., Pettinato R. and Pillot T. 2001 Allele epsilon 4 of apolipoprotein E gene is less frequent in Down's syndrome patient of the Sicilian population and has no influence on the grade of mental retardation. *Neurosci. Lett.* **306**, 129–131.

Benson R. C., Beard C. M., Kelalis P. P. and Kurland L. T. 1991 Malignant potential of the cryptorchid testis. *Mayo Clin. Proc.* **66**, 372–378.

Chew G. and Hutson J. M. 2004 Incidence of cryptorchidism and ascending testes in trisomy 21: a 10 year retrospective review. *Pediatr. Surg. Int.* **20**, 744–747.

Fonkalsrud E. W. 1970 Current concepts in the management of the undescended testis. *Surg. Clin. North Am.* **50**, 847–852.

Ichiyanagi O., Sasagawa I., Kubota Y., Yaguchi H., Suzuki Y. and Nakada T . 1998 Down's syndrome associated with seminoma in undescended testis. *Scand. J. Urol. Nephrol.* **32**, 365–367.

Koolen D. A., Nillesen W. M., Versteeg M. H., Merkx G. F., Knoers N. V., Kets M. *et al*. 2004 Screening for subtelomeric rearrangements in 210 patients with unexplained mental retardation using multiplex ligation dependent probe amplification (MLPA). *J. Med. Genet.* **41**, 892–899.

Kouprina N., Pavlicek A., Noskov V. N., Solomon G., Otstot J., Isaacs W. *et al*. 2005 Dynamic structure of the *SPANX* gene cluster mapped to the prostate cancer susceptibility locus *HPCX* at Xq27. *Genome Res.* **15**, 1477–1486.

Rapley E. A., Crockford G. P., Teare D., Biggs P., Seal S., Barfoot R. *et al*. 2000 Localization to Xq27 of a susceptibility gene for testicular germ-cell tumours. *Nat. Genet.* **24**, 197–200.

Rowland J. S., Barton D. E., Taylor G. R. and UK clinical molecular genetics society HMSN project group 2001 A comparison of methods for gene dosage analysis in HMSN type 1. *J. Med. Genet.* **38**, 90–95.

Rychlik W. and Rhoads R. E. 1989 A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and *in vitro* amplification of DNA. *Nucleic Acids Res.* **17**, 8543–8551.

Salemi M., Calogero A. E., Bosco P., Castiglione R., La Vignera S., Borgione E. *et al*. 2006 Expression of SpanX proteins in normal testes and in testicular germ cell tumours. *Int. J. Androl.* **29**, 368–373.

Salemi M., Calogero A. E., Bosco P., Castiglione R., La Vignera S. and Borgione E. 2006 Expression of SpanX mRNA in testicular germ cell tumours. *Hum. Cell* **19**, 87–90.

Salemi M., Romano C. and Ragusa L. A. 2007 A new 6-bp SOX3 polyalanine tract deletion does not segregate with mental retardation. *Genet. Testing* **11**, 124–127.

Salemi M., Bosco P., Calí F., Calogero A. E., Soma P. F., Galia A. *et al*. 2008 *SPANX-B* and *SPANX-C* (Xq27 region) gene dosage analysis in Sicilian patients with melanoma. *Melanoma Res.* (in press).

Sharp A. J., Locke D. P., McGrath S. D., Cheng Z., Bailey J. A., Vallente R. U. *et al*. 2005 Segmental duplications and copy-number variation in the human genome. *Am. J. Hum. Genet.* **77**, 78–88.

Shaw C. J. and Lupski J. R. 2004 Implications of human genome architecture for rearrangement-based disorders: the genomic basis of disease. *Hum. Mol. Genet.* **1**, 57–64.

Westbrook V. A., Diekman A. B., Klotz K. L., Khole V. V., von

Kap-Herr C., Golden W. L. *et al*. 2000 Spermatid-specific expression of the novel X-linked gene product SPAN-X localized to the nucleus of human spermatozoa. *Biol. Reprod.* **63**, 469–481.

Westbrook V. A., Schoppee P. D., Diekman A. B., Klotz K. L., Allietta M. and Hogan K. T. 2004 Genomic organization, incidence, and localization of the SPAN-X family of cancer-testis antigens in melanoma tumors and cell lines. *Clin. Cancer Res.* **10**, 101–112.

Wang Z., Zhang Y., Liu H., Salati E., Chiriva-Internati M. and Lim S. H. 2003 Gene expression and immunologic consequence of SPAN-Xb in myeloma and other hematologic malignancies. *Blood* **101**, 955–960.

Zendman A. J., Zschocke J., van Kraats A. A., de Wit N. J., Kurpisz M., Weidle U. H. *et al*. 2003 The human *SPANX* multigenic family: genomic organization, alignment and expression in male germ cells and tumor cell lines. *Gene* **309**, 125–133.

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