**CDX2 mutations do not account for juvenile polyposis or Peutz–Jeghers syndrome and occur infrequently in sporadic colorectal cancers**

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**Summary**

Peutz–Jeghers syndrome (PJS) and juvenile polyposis (JPS) are both characterized by the presence of hamartomatous polyps and increased risk of malignancy in the gastrointestinal tract. Mutations of the LKB1 and SMAD4 genes have been shown recently to cause a number of PJS and JPS cases respectively, but there remains considerable uncharacterized genetic heterogeneity in these syndromes, particularly JPS. The mouse homologue of CDX2 has been shown to give rise to a phenotype which includes hamartomatous-like polyps in the colon and is therefore a good candidate for JPS and PJS cases which are not accounted for by the SMAD4 and LKB1 genes. By analogy with SMAD4, CDX2 is also a candidate for somatic mutation in sporadic colorectal cancer. We have screened 37 JPS families/cases without known SMAD4 mutations, 10 Peutz–Jeghers cases without known LKB1 mutations and 49 sporadic colorectal cancers for mutations in CDX2. Although polymorphic variants and rare variants of unlikely significance were detected, no pathogenic CDX2 mutations were found in any case of JPS or PJS, or in any of the sporadic cancers. © 2001 Cancer Research Campaign http://www.bjcancer.com

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Germline mutations of the LKB1/STK11 gene, a serine threonine kinase, on 19p13.3 have been shown to account for the majority of Peutz-Jeghers Syndrome (PJS, OMIM 175200) cases (Hemminki et al., 1998). Nevertheless, up to 50% of PJS cases have no detectable LKB1 mutation and families unlinked to 19p13.3 have been reported (Olischwang et al., 1998). PJS patients develop hamartomas at many sites including the gastrointestinal tract, and the polyps have an arborizing structure with a smooth muscle core. Hamartomas of the GI tract are the main characteristic of Juvenile Polyposis Syndrome (JPS, OMIM 174900), but the polyps differ from those in PJS: the juvenile polyps do not have the characteristic smooth muscle core of PJS polyps, they have a greater inflammatory component, and have expanded mucin-filled cysts. Germline mutations of the SMAD4/DPC4 gene, a downstream regulator in the TGFβ signalling pathway, on 18q21.1, cause JPS, but can only account for about 20–25% of JPS cases (Houlston et al., 1998; Howe et al., 1998). The SMAD4 gene has been shown to act as a tumour suppressor gene not only in JPS (Woodford-Richens et al., 2000) but also in sporadic colon and pancreatic cancers (Thiagalingam et al., 1996; Hahn, 1996 #435). Therefore, it seems likely that genes which may be considered as good candidates for the remaining PJS and JPS cases are those which are involved as part of the stepwise progression of colorectal cancer.

The caudal-type homeobox gene, CDX2 (Genbank accession numbers AF003845/6), encodes a transcription factor which is expressed in the intestine, and is thought to play a role in the differentiation and proliferation of intestinal epithelial cells (Drummond et al., 1997; Lorentz et al., 1997). Mice heterozygous for CDX2 null mutations give rise to a phenotype that includes colonic polyps reported to have features of hamartomas (Chawengsaksophak et al., 1997; Tamai et al., 1999). These hamartomas do not express CDX2, indicating that biallelic inactivation of the CDX2 gene is probably an important step for the growth of hamartomas (Tamai et al., 1999). CDX2 has also been implicated in the development and progression of a subset of human colorectal cancers (da Costa et al., 1999; Mallo et al., 1997; Yagi et al., 1999).

Taken together, these findings indicate that the CDX2 gene may be a good candidate for those cases of PJS and JPS who are not explained by mutations in LKB1 or SMAD4. We have therefore screened the CDX2 gene for germline mutations in 37 JPS patients and 10 PJS patients without known SMAD4 or LKB1 mutations, and for somatic mutations in 49 sporadic colorectal cancers.

**PATIENTS AND METHODS**

Patients were selected who had JPS (five or more juvenile polyps or any number of juvenile polyps and a family history of JPS, plus no clinical features suggestive of other hamartoma syndromes such as Cowden, Gorlin or Bannayan-Zonana syndromes) or PJS (characteristic hamartomas of gastrointestinal tract and classical pigmentation of the lips and buccal mucosa, or with only one of these features in a familial context). Each patient had been screened previously for either SMAD4 (JPS) or LKB1 (PJS)
mutations, but none had been found (Hemminki et al., 1998; Houlston et al., 1998; Ylikorkala et al., 1999). Also chosen for screening were 49 colorectal cancer cell lines (C10, C32, C70, C75, C80, C84, C99, C106, C125, CAC02, COLO201, COLO205, COLO206, COLO320, COLO678, COLO741, CX1, DLD1, GP2D, GP5D, HCA46, HCA7, HCT8, HCT15, HCT116, HRA19, HT29, HT55, LIM1863, LOVO, LS174T, LS180, LS411, LS1034, PC/JW, SKCO1, SW48, SW403, SW480, SW620, SW837, SW948, SW1222, SW1417, T84, VAC04A, VAC04S, VAC05, VAC010). Control samples were derived from an unselected UK population with no known cancer predisposition, but were not matched for age or sex. DNA was extracted from peripheral blood lymphocytes and cell lines using standard methods.

PCR primers for exon-by-exon amplification of CDX2 from genomic DNA (Genbank accession numbers AF003845/6) were designed using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), with the forward and reverse primers both fluorescently dye-labelled (FAM, TET or HEX). PCRs were performed according to the conditions shown (Table 1), and then diluted 1:50 with distilled water. After combining the fluorescent PCR products with an artificial size standard (Tamra 350, PE Applied Biosystems, Warrington, UK) and formamide, F-SSCP analysis was performed using an ABI310 sequencer (PE Applied Biosystems, Warrington, UK), under two different temperature conditions (20°C and 35°C). PCR products were also screened for mutations causing conformational change using the PHAST mini-gel SSCP system at 10°C according to manufacturer's instructions, again using different temperature conditions (Pharmacia, Uppsala, Sweden).

Fragments showing both aberrant and normal migration were re-amplified using non-fluorescently labelled primers, purified using Qiaquick columns (Qiagen, Hilden, Germany) and then sequenced in both forward and reverse orientations using the ABI Big Dye Terminator kit (PE Applied Biosystems, Warrington, UK). Sequencing was performed on a subset of samples (at least 10) with normal SSCP bands to ensure all mutations were detected. No mutations were detected by direct sequencing that were not detected by F-SSCP.

Table 1  Primers and PCR conditions for CDX2. Exon 1 is divided into three parts so suitable fragments size for SSCP were obtained. 'Temp.' indicates the annealing temperature of the PCR reaction and 'Mg2+' shows Mg2+ concentration required.

| Exon | Forward primer | Reverse primer | Temp. (°C) | Mg2+ (mM) |
|------|----------------|----------------|-----------|-----------|
| 1 part 1 | CAGCATGGTGAGGCTCTGC | GCGTAGCCATTCCAGTCT | 55 | 0.5 |
| 1 part 2 | GCCAGCGAACTTGCGAG | GCTTAGGCTGTTGACGAG | 55 | 1 |
| 1 part 3 | AGCCCCGCAGACTACCAT | GCGAGCCTCTGCTTACCTT | 55 | 0.5 |
| 2 | GCCCTCACTTCTTCTTCTCTC | GTCCCAAGCCTGCTCTC | 65 | 2.5 |
| 3 | TTTTCTCCACCTTCTTCCATT | TCAGCCTGGAATTGCTTG | 55 | 2.5 |

Table 2  Frequencies of the polymorphic CDX2 exon 3 alleles in JPS, PJS, colorectal cancer cell lines and a control cohort. The observed frequencies of the respective alleles did not differ significantly between patients and controls (Fisher's exact test, P > 0.3). The genotype frequencies do not differ significantly from Hardy–Weinberg equilibrium in any case (details not shown).

| Patients | Frequency of (t/t) homozygotes (%) | Frequency of (c/t) heterozygotes (%) | Frequency of (c/c) homozygotes (%) |
|---------|----------------------------------|-------------------------------------|----------------------------------|
| Juvenile polyposis | 68 (25/37) | 30 (11/37) | 2 (1/37)  |
| Peutz–Jeghers | 80 (8/10) | 20 (2/10) | 0 (0/10)  |
| CRC cell lines | 72 (35/49) | 22 (11/49) | 6 (3/49)  |
| Controls | 78 (40/51) | 22 (11/51) | 0 (0/51)  |

RESULTS AND DISCUSSION

No pathogenic mutations of CDX2 were found in the 37 JPS cases or in the 49 colorectal cell lines. One of the 10 PJS cases showed aberrant migration for CDX2 exon 3 on SSCP, which upon sequencing showed an A to T base change at nucleotide 941. This change lies in the 3′ untranslated region of the CDX2 gene upstream of the poly A signal, and is not conserved in the mouse CDX2 mRNA (Genbank NM007673). The mutation was not observed in any of the JPS cases, colon cancer cell lines or 100 normal control chromosomes, but its significance must remain doubtful.

A missense polymorphism was detected in exon 3, a TCT to CCT transition at nucleotide 871 which introduces a serine to proline amino acid change at codon 291. Although this might be a potential phosphorylation site, sequence analysis programs detect no evidence of homology to any consensus sequence (details not shown). Mouse Cdx2 has a proline residue at codon 291 and is not known to be polymorphic. The frequencies of the serine and proline alleles were not significantly different in the JPS or PJS patients, in the cancer cell lines from the frequencies in a UK control cohort (Table 2) or from those frequencies previously reported in colorectal cancer (Yagi et al., 1999) (data not shown). These data therefore confirm previous suggestions that S291P is a polymorphism (Wicking et al., 1998), which is not functionally significant for JPS or PJS, although some potential functional significance as a low-penetration cancer predisposition allele cannot entirely be excluded.

The previously reported silent polymorphism at codon 61, a CCG to CCC change (Yagi et al., 1999), was not detected in our study using SSCP, or by direct sequencing of 10 of the cancer cell line DNAs. This may result from population differences between studies. However, being a silent polymorphism the nucleotide change was anticipated not to produce any functional effect.

Thus, despite the Cdx2 mouse knockout developing colonic hamartomas which makes Cdx2 a good candidate for JPS and PJS, we have shown that germline CDX2 mutations do not account for the development of these two hamartoma syndromes. We have assessed linkage to the CDX2 region (13q12.3) in the familial JPS
cases and found no evidence of linkage to this region, again sustaining that CDX2 is probably not important in PJS or JPS (data not shown). The one JPS patient who possessed an A to T transversion in the 3' UTR is unlikely to have their disease attributable to this sequence variant – unless the change contributes directly to mRNA stability or splicing in some unknown fashion. One possible way of determining the role of this variant would be to look for a ‘second hit’ – that is, biallelic inactivation of CDX2 – in the hamartomas of this patient, but no such material was available. It has been suggested that the hamartomas in the knockout mouse resemble heterotopias or intercalations, that is reduplication of gut tissue in the colon, rather than true hamartomas (Beck et al, 1999). If so, it may therefore be unsurprising that no pathogenic mutations were found in two human syndromes where true hamartomas are present.

We have also shown that none of 49 colorectal cancer cell lines possesses a pathogenic mutation of CDX2. Previous reports have all found a low frequency of CDX2 mutations in colon cancers (da Costa et al, 1999; Yagi et al, 1999). In one study, a single colon cancer showed biallelic inactivation of CDX2 and restoration of expression inhibited growth (Wicking et al, 1998). Overall, it remains to ascertain whether this gene has an important role in the development of colorectal malignancies. The genetic aberrations which are responsible for cases of PJS and JPS and not caused by the LKB1 and SMAD4 genes respectively, remain elusive. Although there is debate regarding the existence of a second PJS locus, there is convincing evidence of at least one additional JPS locus. Further screening of candidate genes may be necessary to discover this gene, since the alternative – linkage analysis – will be problematic: most JPS families are too small to exclude SMAD4 linkage and the existence of families with undetectable SMAD4 mutations may confound the detection of critical recombinants.

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