Impact of E27X, a novel CDKN2A germ line mutation, on p16 and p14ARF expression in Italian melanoma families displaying pancreatic cancer and neuroblastoma

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Mutations in the CDKN2A gene underlie melanoma susceptibility in as many as 50% of melanoma kindreds in selected populations, and several CDKN2A founder mutations have been described. Inherited mutations in CDKN2A have been found to be associated with other, non-melanoma cancers including pancreatic cancer (PC) and neural system tumors (NST). Here we report a novel germline mutation in exon 1 of the CDKN2A gene, E27X, which we first detected in melanoma patients living in or originally from a small geographic area bordering Liguria in north-western Italy. A subset of melanoma kindreds positive for this mutation displayed PC and neuroblastoma. E27X generates a premature stop codon, leading to dramatically reduced protein levels of p16 and leaving p14ARF unaltered. As PC and NSTs have been postulated to be preferentially associated with CDKN2A mutations located in exon 2 and/or affecting p14ARF alone, the position of E27X in exon 1 provides interesting insights towards clarifying the mechanisms by which the CDKN2A/ARF locus is involved in cancer predisposition.

INTRODUCTION

The CDKN2A gene (MIM no. 600160), located on chromosome 9p21 (1–3) is the most common high penetrance susceptibility gene identified to date in melanoma families. The mutation rate is ~20% on average, but ranges between 5 and 50% depending on family and population selection (4,5).

CDKN2A codes for two distinct proteins, p16 and p14ARF, which arise from separate first exons (1α and 1β), and are spliced onto the common exons 2 and 3, in different reading frames. Both proteins act as tumor suppressors: p16 acts through the pRB pathway, p14ARF directly stabilizes p53 and also functions in the pRb pathway, presumably impairing both (6–11). Mutational events in the CDKN2A locus can cause loss of function of either or both proteins, so that each may contribute specifically to the development of different types of cancer.

Some CDKN2A-positive kindreds have been found to display other cancers in addition to melanoma. An increased risk of pancreatic cancer (PC) has been established in a
subset of melanoma families worldwide carrying mutations located in exon 2 of the CDKN2A gene and thus predicted to affect the amino acid sequence of the alternatively spliced p14ARF melanoma susceptibility gene (12–18). Although the relationship between the CDKN2A locus and PC remains unclear, an association seems to exist between the location of mutations and occurrence of PC.

Neural system tumors (NSTs) have been reported to be associated with large deletions of CDKN2A/ARF and/or mutations that affect p14ARF only (19–22). Abnormal expression of p14ARF, sometimes coupled with normal p16 protein expression but most often with abnormal p16, has been associated with a poor prognosis in neuroblastoma (23), suggesting that p14ARF plays an important role in the development of this tumor.

Most of the recurrent CDKN2A mutations identified result from single mutational events (24–31). The CDKN2A G101W founder mutation, a missense mutation in exon 2, is one of the most frequent in melanoma families worldwide and is associated with an increased risk of PC (16). G101W is commonly identified in melanoma families from north-western Italy (32) who also display an increased risk of PC. Further, G101W has been detected in a subset of PC patients in a case–control study from the same area (33).

Here we report a novel germline CDKN2A mutation, E27X, which we first detected in melanoma patients living in or originally from a small geographic area bordering Liguria in north-western Italy. We provide evidence of a common founder and investigate the association between PC, neuroblastoma and the E27X mutation.

RESULTS

CDKN2A E27X-positive patients and families

A G>T transversion at nucleotide 79 determining a change from glutamic acid to an amber stop codon at codon 27 of CDKN2A exon 1a (E27X) (Fig. 1A) was detected in three kindreds (Fig. 1B–D) who live in and are originally from a very small area in southern Piedmont, bordering Liguria. The mutation segregated with the disease and was found in all the affected family members tested. None of the 100 geographic controls tested carried the mutation. The FAM_1 proband displayed multiple melanomas, the daughter of the FAM_3 proband was also E27X-positive, was diagnosed with neuroblastoma at the age of 3 and died a few months later.

The E27X mutation was independently identified in a melanoma family attending the Medical Genetics Service at the University of Florence (FAM_4; Fig. 1E) but originally from southern Italy. The family displays four cases of melanoma and three cases of PC. Two of the PCs were reported from southern Italy. The family displays four cases of melanoma and three cases of PC. Two of the PCs were reported from southern Italy; the third patient (NF_3), who had multiple melanomas, was originally from southern Italy. The median age at diagnosis of the E27X mutation-positive cases (six tested and seven inferred) (n = 13) was 35 years. This seems to be lower than the median age at diagnosis (46 years) observed in mutation-positive melanoma patients carrying G101W (n = 63), the most frequent founder mutation in north-western Italy. The difference, however, is not significant and larger numbers of cases are needed. Additionally, as observed for CDKN2A mutations in general, the median age at melanoma diagnosis for both E27X and G101W is substantially lower than the median age at diagnosis in the general Italian melanoma population (57 years) (34, and unpublished data) or in the Province of Genoa (55.8 years) (Cancer Registry data updated to 2000, unpublished data).

Haplotype analysis

All of the E27X-positive patients revealed a genotype/haplotype that was consistent with a single genetic origin for this mutation. Genotypes associated with a conserved haplotype at D9S1749, which may have arisen through replication slippage (Table 1). Alleles that were part of the disease-related haplotype are shown in boldface. Both alleles are indicated for markers for which disease-segregating alleles could not be unequivocally determined (Table 1).

Dating the mutation

Genotype/haplotype data from the seven families with the E27X mutation were used to estimate the origination of the mutation. Maximum likelihood (MLE) results suggest that the E27X mutation was first observed 20 generations ago (two-LOD-unit support interval: 3–63 generations), or ~400 years ago (using a 20-year generation interval) or 600 years ago (using a 30-year generation interval).

Western blot analysis of p16 and p14ARF protein levels in E27X positive cases and controls

Protein analysis by western blot was performed in lymphoblastoid cell lines from mutation-positive and -negative subjects and showed that p16 protein levels were dramatically reduced in cases compared with controls (12%. P < 0.01). In contrast, for p14ARF, there was no evidence for reduction in protein levels and thus no difference in p14ARF levels between the two groups (Fig. 2).

RNA analysis

Given that such low protein levels may be attributable to modification of RNA size, quantity or decay, we performed RNA analysis in E27X-positive lymphoblastoid cell lines and controls. RT–PCR and long-range RT–PCR analysis showed no difference in the PCR product in E27X carriers versus controls in terms of quantity or size, nor the presence of additional products for CDKN2A and exon 1β. Sequencing
Figure 1. (A) Identification of the CDKN2A E27X germline mutation. The heterozygous G>T transversion (N) is indicated by an arrow. (B–E) Melanoma pedigrees carrying the E27X mutation. Age at diagnosis indicates confirmation of cancer diagnosis and is provided below each symbol.

Table 1. 9p21 haplotype conservation in melanoma probands carrying the E27X germline mutation

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Regions of shared haplotype are indicated in italics. Alleles that are part of the disease-related haplotype are shown in boldface. Both alleles are indicated for markers for which disease-segregating alleles could not be unequivocally determined.

aAllele-1, larger allele than the one designated as allele 1 by Pollock et al. (49).
reactions were performed with both the primers used to amplify cDNA (in exon 1α 5′-UTR, exon 1β and a common 3′-UTR) and exonic primers in exon 2, to cover the whole gene and splicing junctions. Sequencing results showed biallelic RNA expression, with no evidence of a second mutation impacting on the wild-type p16 allele. Although the results of long-range RT–PCR product sequencing are not quantitative, heterozygosity for the E27X mutation and for the nucleotide 500G/C polymorphism, previously detected at the genomic level, was confirmed, as was the integrity of the wt exon 1β. The similar height of the peaks at the heterozygous nucleotides in sequencing reactions suggests that no relevant RNA decay result from the E27X mutation for both CDKN2A and ARF (nucleotide 500 polymorphism). The clear baseline of the electropherograms showed that no alternative product, which may have been undetectable by agarose gel analysis, was present, however faint. These results indicate that no gross RNA rearrangements, including exon skipping, nor relevant RNA decay result from the E27X mutation for both CDKN2A and ARF. Rather, this mutation mainly seems to impact on the protein level of p16.

DISCUSSION

The E27X mutation appears to have arisen from a common founder and, after G101W, is the most frequent CDKN2A founder mutation identified to date in Italian melanoma patients and families (32,35–38). To our knowledge, this mutation has not been previously reported outside Italy, with the exception of a somatic occurrence in an oral carcinoma (39) from a patient living in London, UK, and of unknown ethnic origin. Haplotype analysis revealed conservation in all mutation positive cases, and according to our preliminary estimate, E27X first originated 20 generations ago (confidence interval: 3–63 generations). Among the CDKN2A founder mutations whose origin has been estimated (113insArg, V126D, G101W), E27X appears to be the most recent. However, our estimates are limited by the small number of families and imprecise haplotype data.

E27X is the first stop codon CDKN2A founder mutation detected in melanoma families who display PC and NST (neuroblastoma). Our findings are suggestive of an association but require further confirmation given the small numbers presented here.

According to previous results mainly relating to melanoma families that carry CDKN2A founder mutations, PC is preferentially associated with mutations in exon 2, which impair both p16 and p14ARF. A recent meta-analysis of literature data (up to 2002) showed that PC is most frequent (>30%) in families carrying mutations in ankyrin domains 3 and 4 (ankyrin 2 covering the end of exon 1α and the first 20 amino acids of exon2) and less (<10%) in kindreds positive for mutations in ankyrin domains 1 and 2 (40). NSTs have been reported to be associated with large deletions and/or mutations involving p14ARF (19–22,41). However, a recent study on Jewish melanoma kindreds with NST found no alterations at the CDKN2A/ARF locus (42), and a Swedish report similarly failed to identify the common CDKN2A Swedish founder mutation 113insArg in multiple melanoma patients with NST (43). The link between melanoma, PC and NST is therefore still unclear, and the separate and joint contributions of p16 and p14ARF need to be investigated.

Given that E27X is located on exon 1α, it may not affect p14ARF. Additionally, this mutation determines a premature termination codon (PTC) with a putative mutant protein of just 27 amino acids, which is likely to lead to the synthesis of a truncated protein and could determine p16 haploinsufficiency. Indeed, quantitation of p16 and p14ARF by western blot of lymphoblastoid cell lines lysates revealed dramatic p16 protein reduction in mutation-positive versus mutation-negative cases, suggesting far lower residual translational activity than the expected 50%, and no evidence for protein reduction for p14ARF. Therefore, we cannot rule out that the mutant RNA containing the PTC is targeted by nonsense-mediated decay or other mechanisms for rapid degradation (44) and affects translation of the wild-type mRNA.

Besides modifying RNA quantity or decay, nonsense mutations are known to modify RNA size and activate cryptic exonic splice sites. Interestingly, such a site has been described to be activated immediately downstream of codon 27 by a splice site mutation leading to an abnormal transcript with exon skipping effects (45), so it would not be unreasonable to postulate that the E27X mutation actually operates both as a nonsense and as a splicing mutation. In our study,
RT–PCR and sequencing analyses confirmed biallelic expression, as determined by heterozygosity for the E27X mutation and for the nucleotide 500G/C polymorphism. Both RT–PCR and long-range RT–PCR ruled out the presence of atypical transcripts as a result of mutation-derived intron retaining or exon skipping for both p16 and p14ARF.

Overall, our findings suggest that E27X impacts on p16 protein without involving gross RNA rearrangements and that it alters the ratio between p16 and p14ARF without impacting on p14ARF. A combined effect of p16 and p14ARF in tumor development has been previously hypothesized (46) and loss of coordinate expression has been put forward as an alternative mechanism for melanoma susceptibility in 9p21-linked families with no detectable mutations (47). More specifically, Hewitt et al. (46) recently described a germline splice site mutation (g.193) in ARF in a melanoma and breast cancer family from the UK and showed that somatic inactivation of both CDKN2A alleles, and possibly of the wild type ARF allele, is likely necessary for tumor development. The g. 193 and a g.193 + 1 splice site mutation have been recently detected in two other unconnected tumor families from the UK, one of which included a case of brain cancer, and were also found to determine p14ARF splicing (22). Given that these mutations abolish the contribution of the mutant allele, it has been hypothesized that g. 193 and g.193 + 1 reduce the amount of wild-type protein to below a threshold level, thus impairing p14ARF function, either through mRNA instability or through translation of a non-functional protein. The data presented here indicate that a similar hypothesis may be applied to the impact of the mutant E27X on the ratio between p16 and p14ARF and could explain predisposition to multiple tumors by inactivation of both the pRB and the P53 pathways. Tissue expression studies, LOH and quantitative RNA analyses are required to further clarify the role of both proteins in the development of melanoma, PC and NSTs.

Given the small number of families with the E27X mutation, it is difficult to precisely estimate when the mutation likely originated. Reports on greater numbers of families would help to better define the origin and spread of this novel founder mutation. Preliminary analyses within an ongoing PC case–control study have already confirmed germ-line presence of the E27X mutation in two cases coming from the same area in southern Piedmont (unpublished data). In the near future, we hope to assess the contribution of E27X or other mutations involving p16/p14ARF in NST cases from the same area of Italy.

MATERIALS AND METHODS

Patients and families
The Medical Genetics Service at the San Martino Hospital in Genoa routinely provides genetic counseling and testing to self-referred melanoma families with at least two melanomas in the same branch.

Clinically sporadic patients are also tested for mutations in CDKN2A for research purposes within the context of an ongoing retrospective population-based study (Province of Genoa) and of a local hospital-based study. These studies have been approved by the Ethics Committee of the San Martino Hospital/University Clinics and of the NCI in Genoa.

Diagnostic confirmation was obtained for all melanoma cases, except one patient in FAM1 who was only reported by relatives. Confirmations for PC and neuroblastoma were obtained up to first degree relatives of probands. Other cancers in the families were self-reported, and age at diagnosis for these self-reported cancers was not confirmed and thus not indicated.

Mutation detection

The CDKN2A coding region, including splice junctions, the 5′-UTR, the intronic sequence described to contain the IVS2-105 A/G mutation and exon 1β, was entirely sequenced, as well as CDK4 exon 2. The primers used have been previously described (32,33,48). Standard PCR conditions were applied, with an annealing temperature of 60°C for all primer sets. Sequencing reactions were performed using an ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystem) and the products were analyzed on an ABI 3100 DNA sequencer (Applied Biosystem). DNA sequencing was carried out in both directions on independent DNA extractions using the same primers used in the initial PCR amplification.

Haplotype study

Probands and members of E27X-positive families were typed for eight 9p21 markers surrounding CDKN2A (IFNA, D9S736, D9S1749, D9S974, D9S942, D9S1748, D9S1604, D9S171; all primer oligonucleotide sequences are available at http://www.gdb.org). For each primer pair, one oligonucleotide was labeled with a 5′-fluorescent amidite (6′ FAM or HEX). Amplification was carried out using TaqGold (Applied Biosystem) and PCR products were visualized using an ABI 3100 automated DNA sequencer (Applied Biosystem). Allele sizes for all markers (except D9S942, D9S171) are comparable with those previously described by Ciotti et al. (27) and Pollock et al. (49).

Mutation dating

To estimate when the E27X mutation originated, we used a MLE method developed by D. Goldgar (50,51) and previously applied to G101W and V126D (27,28). Briefly, the joint likelihood of the E27X haplotypes was written as a function of the recombination fraction between the disease and each marker, the number of generations (G) since the mutation arose and the mutation rate [0.0006 for all markers except D9S1749 (0.01) and D9S942 (0.002)] and allele frequencies at each marker locus estimated from 36 geographic controls from the area. The MLE method was used to find the value of G that best fitted the pattern of haplotype sharing at the eight marker loci. When haplotypes could not be determined with certainty, all possible haplotypes consistent with the observed multilocus genotypes were considered in the analysis. Approximate support intervals were calculated by finding the value of G on either side of the most likely value that had a > =100-fold (i.e. two-LOD) decrease in likelihood.

Approximate support intervals were calculated by finding the value of G on either side of the most likely value that had a > =100-fold (i.e. two-LOD) decrease in likelihood.
RT–PCR and analysis of CDKN2A transcript in mutation-positive and -negative cases

Lymphoblastoid cell lines were stabilized by immortalization of B lymphocytes isolated from peripheral blood of mutation-positive and -negative patients by Epstein–Barr virus. Total RNA was extracted according to standard procedures (Trizol, Life Technologies). cDNA was then reverse transcribed from RNA using the Advantage RT-for-PCR kit (Clontech). The cDNA was amplified using a forward primer in the CDKN2A 5’-UTR, PCR1-f and a reverse primer in the 3’-UTR, PCR1-R, as previously described (45). Control PCR reactions were carried out using primers to amplify the GAPDH housekeeping gene. A standard PCR protocol was used, with denaturation at 95°C for 40 s, annealing at 60°C for 30 s and extension at 72°C for 2 min. Sequencing of the purified products was performed using both the PCR primers and exonic primers in exon 2 (2BF and 2AR) (2). The same PCR protocol was used to amplify the p14ARF cDNA product using the same primer in 3’-UTR and an exonic primer 1BF (5’-ATGGTGCGCAGGTTC TTGGT-3’). Sequencing was performed with the same primers used to amplify cDNA and exonic primers in exon 2, in order to cover the whole gene.

Long-range PCR of mutant and wild-type transcript

The Gene Amp XL PCR Kit with rTth DNA polymerase enzyme (Applied Biosystems) was used to carry out long-range PCR across the whole CDKN2A gene, using the same primers as described above, with an annealing temperature of 68°C as suggested by the manufacturer. Sequencing was also performed on long-range PCR products using both exonic primers (exon 2) and the same primers used to amplify both cDNA products at the CDKN2A locus.

Western blot analysis

Lymphoblastoid cell lines were stabilized by immortalization of B lymphocytes isolated from peripheral blood of mutation-positive and -negative patients by Epstein–Barr virus. An equal number of cells (2 × 10⁷) was counted and cell extracts were obtained by lysing the cells in sample buffer (10% glycerol, 1 mM EDTA, 0.15 M NaCl, 2.5 mM dithiothreitol, 1 mM Na₃VO₄, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 1 mg/ml pepstatin, 1 mM PMSF, 0.5%NP-40 and 20 mM HEPES). Bradford assays were also performed to determine total protein concentration and 40 μg of protein lysate was denatured at 95°C for 5 min and loaded within each well onto 12% sodium dodecyl sulfate (SDS)–polyacrilamide gels. Gels were run at constant voltage (150 V) for 1 h and wet transfer was performed for 2 h at constant voltage (100 V) onto nitrocellulose membranes. The membrane was then blocked for 10 min in 10% milk and then washed in 0.1% TBST three times for 20 min each. Expression of α-tubulin was monitored as internal control to verify equivalent protein loading. The membranes were sectioned to separate the region of the proteins of interest (p16 and p14ARF) from the internal control, then incubated overnight at 4°C with primary antibodies for either p16 (G175-405 BD BioSciences, Pharmingen), p14ARF or α-tubulin (TU-02, Santa Cruz Biotechnoloy), diluted 1:1000 and 1:500, respectively, in 0.1% milk TBST. Membranes were washed three times, incubated with the corresponding HRP-conjugated secondary antibody (1:5000), washed again and incubated with chemiluminescence detection agent (ECL, Amersham), after which film was exposed for detection.

At least two separate extracts from three mutation-positive cases and three mutation-negative controls were cemented. Extracts from the A2058 melanoma cell line (52) were also loaded as positive control for p16 and negative for p14ARF (as it harbors a deletion confined to exon 1B). Prestained SDS–PAGE Standards Low Range (BioRad) were included.

Image analysis

The bands were scanned both for α-tubulin and p16. The band intensity was quantitated by an image analysis software (Leica Q500IMC) and normalized on the level of α-tubulin. Values are means and standard error deviation.

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Conflict of Interest statement. None declared.

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