

ONLINE MUTATION REPORT

Extending the p16-Leiden tumour spectrum by respiratory tract tumours

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Familial atypical multiple mole melanoma (FAMMM; OMIM #155601) is characterised by the familial occurrence of melanoma of the skin in combination with multiple atypical precursor naevi.¹⁻⁴ The disease is inherited as an autosomal dominant trait, with germline mutations in the *p16* (*CDKN2A*) gene having been reported in at least a quarter of FAMMM families. Previously, we reported an increased risk of pancreatic carcinoma in Dutch FAMMM families with a 19 bp deletion in exon 2 of the *CDKN2A/p16* gene (*p16-Leiden*; OMIM #600160.0003).⁴

Recently a patient with three carcinomas of the pharynx and oral cavity with a germline heterozygous *p16-Leiden* mutation was reported.⁵ All three tumours showed inactivation of the retained wild type allele, with the somatic event being aberrant promoter methylation. Two other reports also described the occurrence of head and neck or oral squamous cell carcinomas (OSCC) in families with different *p16* germline mutations.^{6,7} A relationship between *p16* germline mutations and breast cancer has also been suggested, although in the families studied, *BRCA1* and *BRCA2* mutations were not excluded.^{8,9}

We studied a FAMMM family (EMC13769; fig 1) with co-segregation of the *p16-Leiden* germline mutation, with an extraordinary number of tumours comprising OSCCs, lung tumours, breast carcinomas, and colorectal carcinomas. We determined the mutation status in the various patients and investigated by loss of heterozygosity (LOH) analysis of the wild type allele in the tumours, in combination with immunohistochemistry, whether a causal relationship exists between the *p16-Leiden* mutation and the development of the different tumour type. Insufficient tissue was available for methylation studies. We additionally studied four breast tumours and four lung tumours from eight other patients (from seven other families), all of whom carried a germline *p16-Leiden* mutation.

MATERIALS AND METHODS

Patients

Blood samples and/or paraffin embedded tumour samples were obtained for DNA solution from available subjects that had developed a carcinoma, to determine their *p16-Leiden* mutation status. Unavailable subjects with *p16-Leiden* positive offspring were classified as "obligate carriers". Informed consent was given by family members themselves or by their relatives, in case of deceased subjects. Tumours were pathologically verified whenever possible.

Tumour analysis

Paraffin embedded tumour tissues were obtained, and revision of histology was performed. Areas of highest tumour density were selected for further molecular analysis. Serial sections were produced for immunohistochemical analysis.

Key points

- We studied eight different familial atypical multiple mole melanoma families with co-segregation of a *p16-Leiden* germline mutation.
- One family harbours an extraordinarily high number of tumours, comprising, breast, lung, and colon cancers, and oral squamous cell carcinomas (OSCC). In this family it seems that at least three of four lung cancer patients (one unknown), both OSCC patients, and only one of five individuals with breast cancer (two unknown) were carrying the *p16-Leiden* germline mutation. Immunohistochemical testing for p16 was performed and loss of heterozygosity (LOH) of the *p16-Leiden* wild type allele was analysed in different tumours. Additionally, four breast carcinomas and four lung tumours of eight *p16-Leiden* mutation positive patients from the seven remaining families were analysed.
- Immunohistochemistry of p16 was negative in all four analysed lung carcinomas. LOH of the wild type *p16* allele was present in one of three carcinomas tested. In both OSCCs, p16 immunohistochemistry was negative and LOH of the wild type allele was present in the one case analysed. Furthermore, immunohistochemistry of p16 was negative in one of five analysed breast tumours of mutation positive patients and only this tumour showed LOH of the wild type *p16* allele.
- Our results suggest that the *p16-Leiden* germline mutation may be involved in susceptibility to lung cancer and OSCC development in some patients. There is no evidence for a dominant role of the *p16-Leiden* germline mutation in the development of breast cancer, although an interaction with as yet unidentified modifying factors cannot be ruled out.

DNA isolation

Genomic DNA of normal and tumour tissue was isolated from formalin fixed paraffin embedded material, resuspended in 96 µl of PK-1 lysis buffer (50 mmol/l KCL, 10 mmol/l Tris pH 8.3, 2.5 mmol/l MgCl₂, 0.45% NP40, 0.45% Tween 20, 0.1 mg/ml gelatine) containing 5% Chelex beads (Biorad, Hercules, CA, USA) and 5 µl proteinase K (10 mg/ml), and incubated for 12 h at 56°C. The suspension

Abbreviations: AIF, allelic imbalance factor; FAMMM, familial atypical multiple mole melanoma; LOH, loss of heterozygosity; OSCC, oral squamous cell carcinomas

was incubated for 10 minutes at 100°C, centrifuged, and the supernatant carefully decanted.

Polymerase chain reaction amplification

The *p16-Leiden* deletion comprises 19 bp and removes nucleotides 225–243 of exon 2.¹⁰ Genomic DNA from tumour and normal tissue was subjected to PCR amplification using labelled primers containing the 225–243 region; *p16*-forward-TET M1 (tumour) or FAM M1 (normal), sequence 5'-ATGATGGGCAGCGCCCGAGT-3' and *p16*-reverse A2, sequence 5'-ACCAGCGTGCCAGGAAG-3' (Life Technologies). The total volume per reaction was 12 µl including 5 µmol of each primer (stock forward and reverse primer), a mix of 0.25 µl dNTP (10 mmol/l), 1.2 µl magnesium chloride (20 mmol/l), 1.2 µl bovine serum albumin (1 mg/ml), 1.2 µl *AmpliTaq* Gold buffer (without MgCl₂) and 0.25 µl *AmpliTaq* Gold DNA polymerase, 10 ng of normal or tumour DNA, and H₂O. The following conditions were used: 33 cycles of 1 minute at 96°C, 2 minutes at 55°C, 1 minute at 72°C, and a delayed extension step of 7 minutes at 72°C in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Mixtures of 24 µl dionised formamide, 1 µl TAMRA 500 (Applied Biosystems) and 1.2 µl of PCR product were run on a ABI 310 Genetic analyser (Applied Biosystems) for 20 minutes with run profile GS STR POP4 (1.0 ml) C and analysed with Genescan 3.1 computer software (Perkin-Elmer Corp).

Loss of heterozygosity analysis

Owing to the 19 bp deletion, we could specifically analyse the fate of the wild type allele in terms of LOH. Analysis of LOH was possible when both normal and tumour tissue was available. LOH was scored when there was loss of intensity of one allele in the tumour sample with respect to the matched wild type allele from normal tissue. The quotient of the peak height ratios from normal and tumour DNA served as the allelic imbalance factor (AIF)—that is, the ratio of the peak height at 101 bp of the deleted allele and the peak height at 120 bp of the wild type allele. The threshold for allelic imbalance was defined as 40% reduction of one allele, agreeing with an AIF of ≤ 0.59 or > 1.3 . The threshold for retention was defined to range from 0.76 to 1.3 as previously

empirically determined.¹¹ AIFs of 0.60–0.75 and 1.3–1.69 were considered to belong to a so-called grey area, for which no definitive decision has been made.

Immunohistochemical testing for *p16*

Tissue sections (4 µm) were prepared on APES coated slides, and dried overnight in a 37°C oven. Sections were deparaffinised in xylene (3×5 minutes). Endogenous peroxidase was blocked by incubation in methanol/H₂O₂ 0.3% for 20 minutes and sections were rehydrated with ethanol and distilled water. Antigen retrieval for *p16* immunostaining was performed by microwaving in boiling 0.01 mol/l sodium citrate buffer (pH 6.0) for 10 minutes. After cooling for 2 hours and washing (2×5 minutes) in PBS, the sections were incubated overnight at room temperature with mouse anti-human *p16* (1:500, clone JC8; Neomarkers Fremont, CA, USA) with tonsil tissue as positive control. Sections were subsequently washed (3×5 minutes in PBS) and incubated (30 minutes) with biotinylated secondary antibody in PBS/BSA 1%, washed (3×5 minutes in PBS) and incubated (30 minutes) with a horseradish peroxidase/streptavidin complex (SABC). Diaminobenzidine-tetrahydrochloride (DAB) was used as a chromogen, followed by counterstaining with haematoxylin. As a negative control, the primary antibody was omitted. Expression was scored by microscopic examination. Loss of *p16* expression was scored when nuclei of tumour cells stained negative and nuclei of normal (stromal) cells stained positive (internal positive control).

BRCA1 and BRCA2 mutation screening

As described above, we were able to obtain tumour material of five *p16*-Leiden carriers with breast cancer. Three (NFDHT 1–3, table 1) had no first or second degree relative with breast cancer. The other two (EMC 13769 No 50 and LUMC 152, table 1) had several relatives with breast cancer diagnosed before the age of 60 years. Complete *BRCA1* and *BRCA2* mutation analysis was performed in the suspect families (EMC 13769 and LUMC 152) and found to be negative. We screened for germline mutations frequently detected in the Dutch population. Protein truncation tests¹² were also performed for PCR fragments of exon 11, and denaturing gradient gel electrophoresis was performed for the remaining

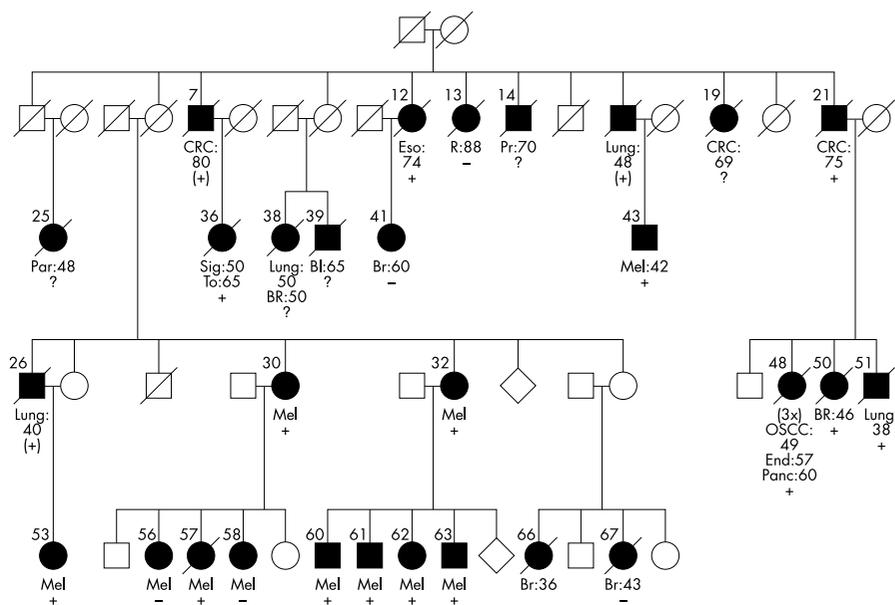


Figure 1 Pedigree of the family EMC13769. Subject number appears above the symbol, age of diagnosis follows the diagnosis. Mel, melanoma; OSCC, oral squamous cell carcinoma. Cancer of the: Bl, bladder; Br, breast; CRC, colorectum; Eso, oesophagus; End, endometrium; Lung, lung; Panc, pancreas; Par, parotid gland; Pr, prostate; R, rectum; Sig, sigmoid; To, tongue. +, *p16*-Leiden positive; -, *p16*-Leiden negative; (+), obligate carrier; ?, *p16*-Leiden carrier status unknown.

Table 1 Results of LOH and immunohistochemical analysis in *p16-Leiden* mutation carriers

Family	Subject no.	Anatomical site	Age at diagnosis (years)	p16-IHC	Internal control	Tumour (%)	LOH
Tumours originating in the lung and oral cavity							
EMC13769	36	OSCC (Tongue)	65	–	–		NA
	48	OSCC (1X)	49	–	+	>50	Yes
	51	Lung (adenocarcinoma)	38	–	+	>50	Yes
NFDHT4	1	Lung (SCC)	61	NA			A
NFDHT4	2	Lung (SCC)	48	–	NP		NA
NFDHT5		Lung (carcinoid)	46	–	+	70–80	R
NFDHT6		Lung (SCC)	56	–	+		NA
Tumours originating in the breast							
EMC13769	50	Breast*	46	+		>30	R
LUMC152		Breast*	41	–	+	50–60	Yes
NFDHT1		Breast	42	+		30	R
NFDHT2		Breast	47	+†		30	R
NFDHT3		Breast	46	+†			NA
Tumours originating in the digestive tract							
EMC13769	21	Colon‡	75	–	+	<30	R
	36	Sigmoid	52	+		>30	R

EMC, Erasmus MC; LUMC, Leiden University Medical Center; NFDHT, Netherlands Foundation for the Detection of Hereditary Tumours; OSCC, oral squamous cell carcinoma; SCC, squamous cell carcinoma.

–, No staining of tumour cells or internal control cells; NP, no internal control cells identified; R, retention of the wild type allele; A, ambiguous; NA, not analysed.

*BRCA1 and BRCA2 tested negative; †few positive tumour nuclei; ‡microsatellite instability analysis: immunohistochemistry for MLH1, MSH2, and MSH6 positive.

exons and exon/intron junctions of *BRCA1* and *BRCA2*. Additionally we screened for the deletions of exon 13 (3.8 kb) and exon 22 (510 bp) of *BRCA1*.¹³

Microsatellite instability

Microsatellite instability was analysed in a diagnostic setting as previously described using markers D2S123, D5S346, D17S250, BAT25, BAT26, and BAT40,¹⁴ and immunohistochemical testing for *MLH1*, *MSH2*, and *MSH6* was performed.¹⁵

RESULTS

Lung cancer

We analysed four different *p16-Leiden* families (table 1, fig 1) with one or more cases of lung cancer. Family EMC13769 (fig 1) harbours four cases of lung cancer. One subject was a proven carrier of a germline *p16-Leiden* mutation (subject 51), two subjects are obligate carriers, and the *p16-Leiden* carrier status remains unknown for one (subject 38). The p16 immunohistochemistry analysis in the tumour of subject 51, a smoker, tested negative, and LOH of the wild type allele was found. The three other (NFDHT) families harbour 4 *p16-Leiden* mutation carriers with documented lung cancers. The immunohistochemistry analysis for p16 was negative in three analysed lung tumours. LOH of the wild type allele was ambiguous in one tumour, and in one tumour a (carcinoid) retention was found (table 1). In the other two tumours no normal tissue was available to perform the analysis.

Oral squamous cell carcinoma (OSCC)

Two subjects of family EMC13769 had a tumour originating in the oral cavity—that is, one tongue carcinoma (subject 36 at 65 years of age) and one subject with three primary OSCCs (subject 48 at 49 years). Immunohistochemical analysis of the tongue carcinoma was negative for p16 but lacked an internal positive control, and LOH analysis was not possible. Immunohistochemical analysis of the one of the three OSCCs from subject 48 (fig 1) tested negative for p16, and LOH of the wild type allele in this tumour was found (table 1).

Breast cancer

We analysed five families with breast cancer. Family EMC13769 shows five cases of breast cancer. Only one was carrying the *p16-Leiden* mutation (subject 50). Germline mutations in *BRCA1* & *BRCA2* were excluded for subjects no 41, 50 and 67. The p16 protein in the tumour from EMC13769 subject 50 stained positive and no LOH was found (table 1). Of the four additional typed breast carcinomas from *p16* mutation carriers from the families LUMC 152 and NFDHT 1–3 (table 1), only one showed expression loss of the p16 protein with LOH of the wild type allele, although in two of four other analysed breast carcinomas only a few tumour nuclei stained positive (with the retention of the *p16* wild type allele in one, the other not tested).

Digestive tract

Family EMC13769 harbours six cases of carcinomas of the digestive tract. Of the two tumours analysed (both patients had a germline *p16-Leiden* mutation), one tumour stained positive and one negative. Neither showed LOH (table 1), nor microsatellite instability (microsatellite stable phenotype of the tumours with normal expression of *MLH1*, *MSH2*, and *MSH6*).

DISCUSSION

All lung and oral cavity tumours studied developed (most likely) in *p16-Leiden* mutation carriers. For two persons we cannot rule out the possibility that the *p16-Leiden* germline mutation in their offspring came from the non-bloodline spouses. However, as this family does not come from the “Dutch region” where multiple *p16-Leiden* mutation carriers have been identified, we think that they are most probably obligate carriers of the same *p16-Leiden* mutation. The age of onset in most patients is unusually young and abrogation of p16 seems present in all analysed cases (4/4), a ratio that seems higher than that encountered in sporadic lung cancer (36–45%).¹⁶ The *p16-Leiden* mutation might therefore indeed predispose carriers to an increased risk of lung and oral cavity carcinomas. With respect to lung cancer, this is supported by two other important observations. Firstly, an increased

cumulative risk of developing lung cancer in male *p16-Leiden* mutation carriers was found compared with the general Dutch population (14.3% v 8.9%).⁴ Secondly, *Cdkn2a* is the most likely candidate for the lung tumour susceptibility locus *pulmonary adenoma progression gene 1 (Papg1)* in mice.^{17–18} *Papg1* has been mapped to a 1.5 cM segment on chromosome 4, which contains the *Cdkn2a* gene that encodes p16^{INK4a}. *Cdkn2a* is polymorphic between the lung tumour resistant mouse strain BALB/cJ and the lung tumour susceptible A/J strain, and the resistant allele is preferentially lost in lung tumours of p16^{INK4a} heterozygous mice. Additionally, germline deletion of the gene in mice leads to increased tumour size and notable histological signs of malignant progression.¹⁷

Sufficient information on the smoking habits of most subjects in our study was lacking. However, smoking may have contributed to the unusually early age of onset of three tumours, although one of the tumours is classified as an adenocarcinoma, a type not typically associated with smoking.

Our study does not provide evidence for a dominant role of *p16-Leiden* in the development of breast cancer. Breast cancer seems also statistically not increased in our cohort studied.⁴ However, in view of the early onset of breast cancer in our *p16-Leiden* positive cases, we cannot rule out a role for the gene in tumour progression, either due to haploinsufficiency or total abrogation of *p16* as seen in one of our cases (LUMC152). Recently, it has been postulated for other genes that mutation or loss of a single allele may be sufficient to play an important role in progression towards cancer.¹⁹ Furthermore, an interaction with as yet unidentified modifying factors (genetic and/or environmental) has yet to be elucidated.

Both analysed tumours from the digestive tract showed no LOH; however, one stained negative. In this case methylation might have inactivated the wild type allele, which is a frequent event in sporadic colon cancer.²⁰ The role of the *p16-Leiden* germline mutation in the development of colon cancer needs further research.

In conclusion, the *p16-Leiden* mutation not only seems to predispose to melanoma and pancreatic tumours but also to head and neck tumours^{5–7}, and tumours of the lung in some families. Promoter methylation⁵ or loss of the wild type allele seems to be the mechanism for the “second genetic hit”. Clinical criteria for *p16* germline mutation screening should be adapted accordingly.

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ELECTRONIC-DATABASE INFORMATION

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for FAMMM (OMIM 155601) and *p16-Leiden* (OMIM 600160.0003)).

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