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Development of esophageal squamous cell cancer in patients with FAMMM syndrome: Two clinical reports

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ABSTRACT

Familial atypical multiple mole melanoma (FAMMM) syndrome is a hereditary syndrome characterized by multiple dysplastic nevi and melanoma. Patients with FAMMM may have a heterozygous, inactivating, pathogenic germline variant in the *CDKN2A* gene, especially the NM_000077.4: c.225_243del19 (p.p75fs) variant, also known as p16-Leiden variant. Patients with this variant are at high risk for developing melanomas and pancreatic cancer due to somatic inactivation of the wild-type *CDKN2A* allele. The combination of an inactivating germline *CDKN2A* mutation and somatic inactivation of the wild-type *CDKN2A* allele in the same cell results in tumor formation. It has been suggested that carriers of a germline *CDKN2A* mutation are also at increased risk for several other cancer types, including esophageal cancer. Here, we describe two unrelated patients with the p16-Leiden variant who developed esophageal squamous cell cancer. Evidence of loss of the wild-type *CDKN2A* allele was obtained in the tumor tissue of both patients indicating biallelic inactivation of p16 in the tumor cells. These results suggest that these patients developed esophageal squamous cell cancer in the context of FAMMM syndrome.

1. Background

Tumor suppressor genes represent an important class of genes. Their products either repress cell proliferation or promote apoptosis. Therefore, inhibition of tumor suppressor genes can result in excessive cell proliferation or diminished apoptosis and thereby drive oncogenesis (Sherr, 2004). An example of a tumor suppressor gene is *CDKN2A*, which resides at locus 9p21. This gene encodes for two proteins p16INK4a and p14ARF. These proteins are involved in the p16-retinoblastoma and p14ARF-p53 pathways and promote G1 to S-phase transition in the cell cycle, cell cycle arrest and apoptosis (Ruas and Peters, 1998; Serrano et al., 1996; Sherr, 2001). Germline pathogenic variants in *CDKN2A* are found in families with familial atypical multiple mole melanoma (FAMMM) syndrome, characterized by multiple dysplastic nevi and melanoma (Lynch et al., 1981, 2002; Bartsch et al., 2002; Smeds et al., 2002; Rulyak et al., 2003; Harinck et al., 2012; Zhen et al., 2015). Apart from melanomas, pathogenic *CDKN2A* variants, especially the NM_000077.4: c.225_243del19 (p.p75fs) variant (p16-

Leiden variant) also cause an increased pancreatic cancer risk. A germline pathogenic *CDKN2A* variant is inherited in an autosomal dominant way (with incomplete penetrance). For biallelic inactivation of the *CDKN2A* gene and thus, the p16 tumor-suppressor protein, a “second-genetic-hit” according to the two-hit hypothesis of Knudson is necessary in the tumor (Knudson, 1971). In addition to melanoma and pancreatic cancer, it has been suggested that patients with a pathogenic *CDKN2A* variant could be at increased risk for other malignancies, such as head and neck cancer and lung cancer (Bartsch et al., 2002; Bergman et al., 1990; Goldstein et al., 1995; Vasen et al., 2000; Borg et al., 2000; Middlebrooks et al., 2019). Here, we describe two patients with FAMMM syndrome, a germline *CDKN2A* mutation (p16-Leiden variant) and esophageal squamous cell carcinoma (ESCC) in whom detailed genomic analysis was performed to study whether or not the esophageal tumor developed in the context of FAMMM syndrome. Moreover, we reviewed published reports on esophageal cancer in FAMMM syndrome.

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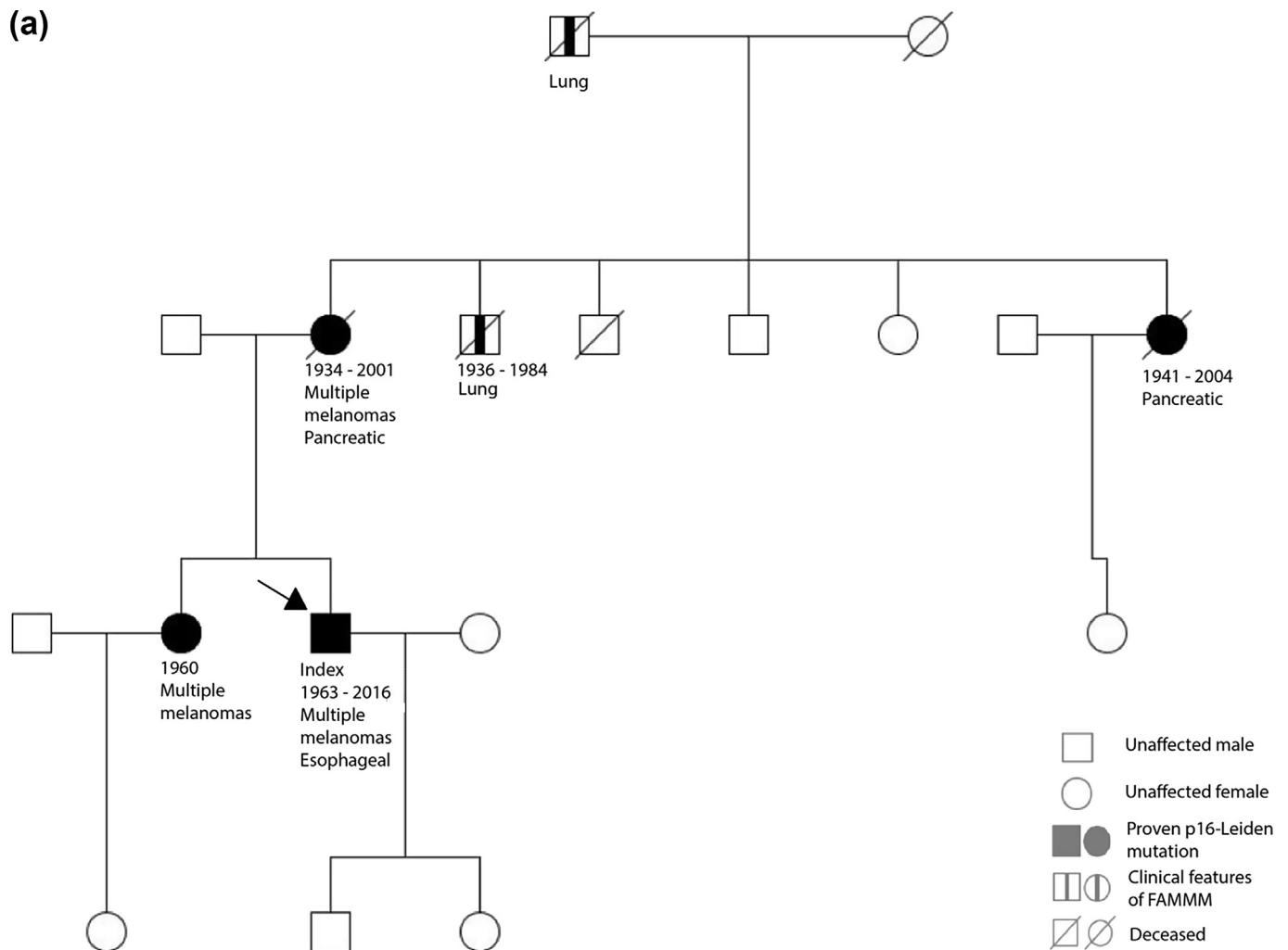


Fig. 1a. Family tree of the first FAMMM patient in whom genomic LOH-analysis was performed (marked by an arrow) with four proven carriers of the p16-Leiden variant in *CDKN2A*. Two additional members of the family had clinical features of FAMMM.

2. Patients and methods

Written informed consent for analysis and publication was obtained from both patients.

2.1. Patient one

A 51-year old man with ESCC was referred to Erasmus University Medical Center, Rotterdam, the Netherlands. The patient has a medical history of dysplastic nevi. Between age 28 and 51 he had developed six melanomas in non-sun exposed areas. He had smoked cigarettes for thirty years (one pack per three days), but had quit smoking three years before diagnosis. He did not drink alcohol. His family history revealed that his sister had multiple melanomas, his mother multiple melanomas and pancreatic cancer, his aunt pancreatic cancer, his uncle lung cancer, and his grandfather lung cancer (Fig. 1a). The patient, his sister, his mother and his aunt were carriers of the p16-Leiden variant in the *CDKN2A* gene, as had been determined previously.

After standard diagnostic work-up following endoscopy with histological biopsies, consisting of endo-ultrasonography, thoracic and abdominal CT and FDG-PET-CT, the patient was staged with cT3N1M0 ESCC. He was scheduled for neoadjuvant chemoradiotherapy, followed by surgical resection (van Hagen et al., 2012). During surgery the tumor appeared unresectable due to infiltration of the aorta. The patient refused palliative chemotherapy and was referred for best supportive

care. He died six months later as a result of disease progression.

2.2. Patient two

A 58-year old female with ESCC was referred to Elisabeth – Tweesteden Hospital in Tilburg, the Netherlands. She had developed four melanomas between 32 and 52 years of age and ovarian cancer at age 47. She used to drink alcohol daily for several years, but quit drinking two years before the current diagnosis. She had never smoked. The patient has two daughters and a son, of whom the two daughters developed melanomas at age 13 and 21 years. Furthermore, two sisters developed melanomas at age 28 and 49. Her mother developed and died from breast cancer at age 53 and one uncle and two male cousins developed melanomas (Fig. 1b). Both the patient herself and her oldest daughter were carriers of the same p16-Leiden variant in *CDKN2A*, as had been determined previously.

Standard diagnostic work-up after endoscopy with histologic biopsies consisted of endo-ultrasonography, thoracic and abdominal CT and FDG-PET-CT and the patient was staged with cT3N0M0 ESCC. She was scheduled for neoadjuvant chemoradiotherapy, followed by minimally invasive esophagectomy; the tumor was microscopically radically resected. Currently, patient is in good health and still in follow-up.

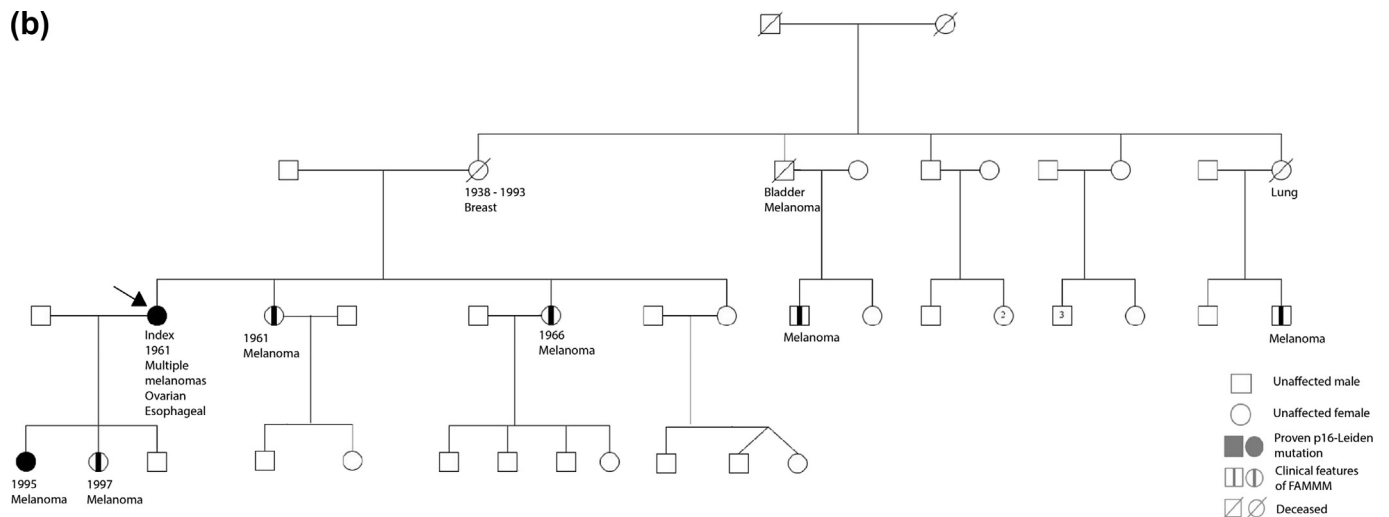


Fig. 1b. Family tree of the second FAMMM patient in whom genomic LOH-analysis was performed (marked by an arrow) with two proven carriers of the p16-Leiden variant in *CDKN2A*. Five individuals had clinical features of FAMMM in whom the mutation was not confirmed.

2.3. Genomic analysis

From both patients, DNA was isolated from formalin fixed an paraffin embedded (FFPE) tissue specimens. Tissue areas composed of normal cells and of a high percentage (> 80%) of tumor cells were manually microdissected from hematoxylin-stained sections (Fig. 2). DNA was extracted using proteinase K and 5% Chelex resin. DNA concentrations were measured using the Qubit 2.0 Fluorometer with the Qubit dsDNA Assay Kit. Next generation sequencing (NGS) of tumor and normal DNA was performed with a custom made primer panel analyzed on an Ion Torrent S5XL-sequencer using suppliers' materials and methods. The custom made primer panel (primer sequences available upon request) was designed using Ion Ampliseq Designer 2.2.1. With this panel, libraries were created using the Ion AmpliSeq Library Kit 2.0–384 LV. Template was prepared using the Ion Chef with the Ion 520- and 530-kit chef. Sequencing was performed on an Ion 530 chip with the Ion PGM S5 Sequencing Kit. Data were analyzed with Variant Caller v5.6. All products used were produced by Thermo Fisher Scientific, Waltham, Massachusetts, United States of America.

2.4. Literature review

We searched Embase, Medline and Google Scholar for individuals

with FAMMM syndrome and esophageal cancer using “esophageal cancer” and “familial multiple-mole melanoma syndrome” as keywords. A detailed description of the search strategy and flowchart is summarized in [Supplementary Figs. 1a and 1b](#).

3. Results

By genomic analysis, the previously identified germline variant in *CDKN2A* exon 2 (p16-Leiden mutation, NM_000077.4: c.225_243del19 (p.p75fs) variant) was found in the esophageal tumors and in the normal tissue of both patients (Fig. 3). This mutation variant was previously submitted in the ClinVar database with access number VCV000182411. Via NGS, a variant allele frequency of 81% and 76% was detected in the esophageal tumor for patient one and two, respectively. In normal tissue, a variant allele frequency was detected of 54% and 64% for patient one and two, respectively. These results indicate relative loss of the wildtype allele in both tumor DNA samples. In addition, loss of heterozygosity (LOH) near and in the *CDKN2A* locus was unambiguously demonstrated with NGS-based single-nucleotide polymorphism (SNP) analysis in both patients (Fig. 4a–c) (Dubbink et al., 2016). As depicted in [Supplementary Fig. 1](#), two previous articles described the presence of FAMMM patients with esophageal cancer (Lynch et al., 2002; Middlebrooks et al., 2019).

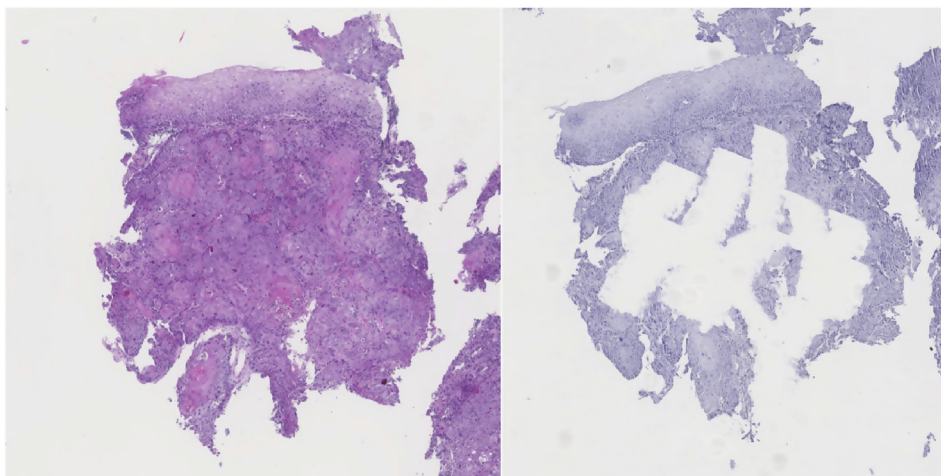


Fig. 2. Hematoxylin and Eosin stainings for patient 2 both before (left) and after (right) microdissection. After pathological examination of these biopsies, patient was diagnosed with squamous cell carcinoma of the esophagus.

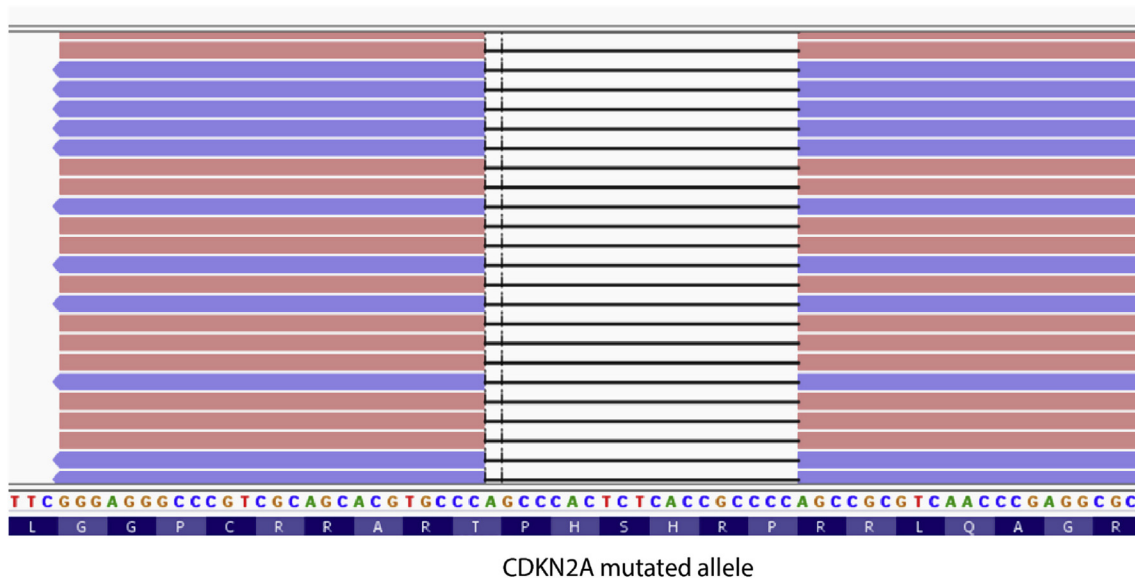


Fig. 3. Sequencing analysis of tumor tissue in patient 2. Sequencing analysis results on the Ion GeneStudio S5 PrimeTorrent Personal Genome Machine for *CDKN2A* exon 2. Each horizontal line represents an individual read of different copies of the amplified sequence; each pink read represents an individual forward strand and each blue read represents an individual reverse strand. The black lines represent the deletions. The A, C, G and T represent the four nucleotides in the sequence and the corresponding amino acids are noted underneath. The example in this figure clearly shows the p16-Leiden NM_000077.4: c.225_243del19 (p.p75fs) variant.

4. Discussion

To our knowledge, this is the first report of FAMMM patients with the p16-Leiden germline variant in *CDKN2A*, who developed ESCC. The SNP analysis of both tumors indicated loss of the *CDKN2A* locus

(Dubbink et al., 2016). In addition, the variant allele frequency of the *CDKN2A* germline mutation in both tumors (patient 1 and patient 2; 81% and 76%, respectively) indicated that this loss was of the wildtype allele with conservation of the germline mutant *CDKN2A* allele. In both tumors, obviously, biallelic inactivation of the *CDKN2A* gene occurred.

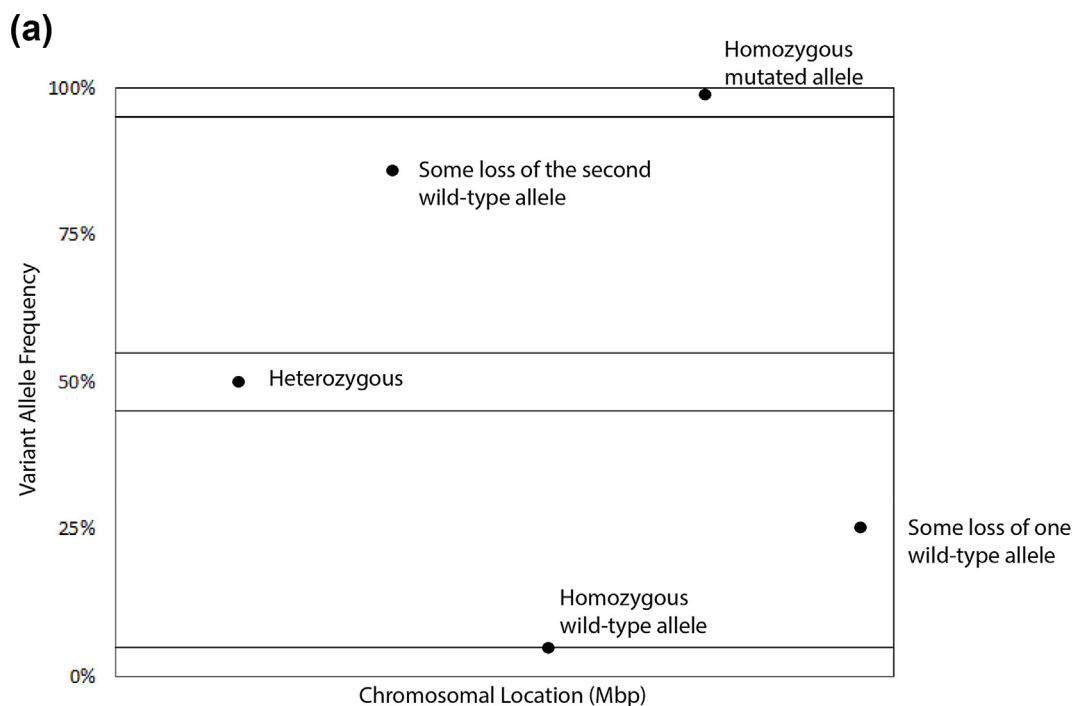


Fig. 4a. Overview on possible locations after NGS-based variant allele frequency analysis. Explanatory figure showing the possible outcomes in NGS-based variant allele frequency analysis. Ordinarily, the X-axis represents the location on the chromosome in million base pairs (Mbp) and the Y-axis represents the variant allele frequency. A variant allele frequency between 0 and 5% indicates a homozygous wild-type allele since nearly no variant allele is detected; a variant allele frequency between 95 and 100% indicates a homozygous mutation of both alleles since (nearly) all of the alleles are of the variant type. A variant allele frequency between 45 and 55% indicates heterozygosity since roughly half of the alleles is of the variant type. A variant allele between 5 and 45% indicates that some of one wild-type allele is lost and predominantly wild-type alleles are still present, a variant allele frequency between 55 and 95% indicates that some of the second wild-type allele is also lost and predominantly the mutated allele is present.

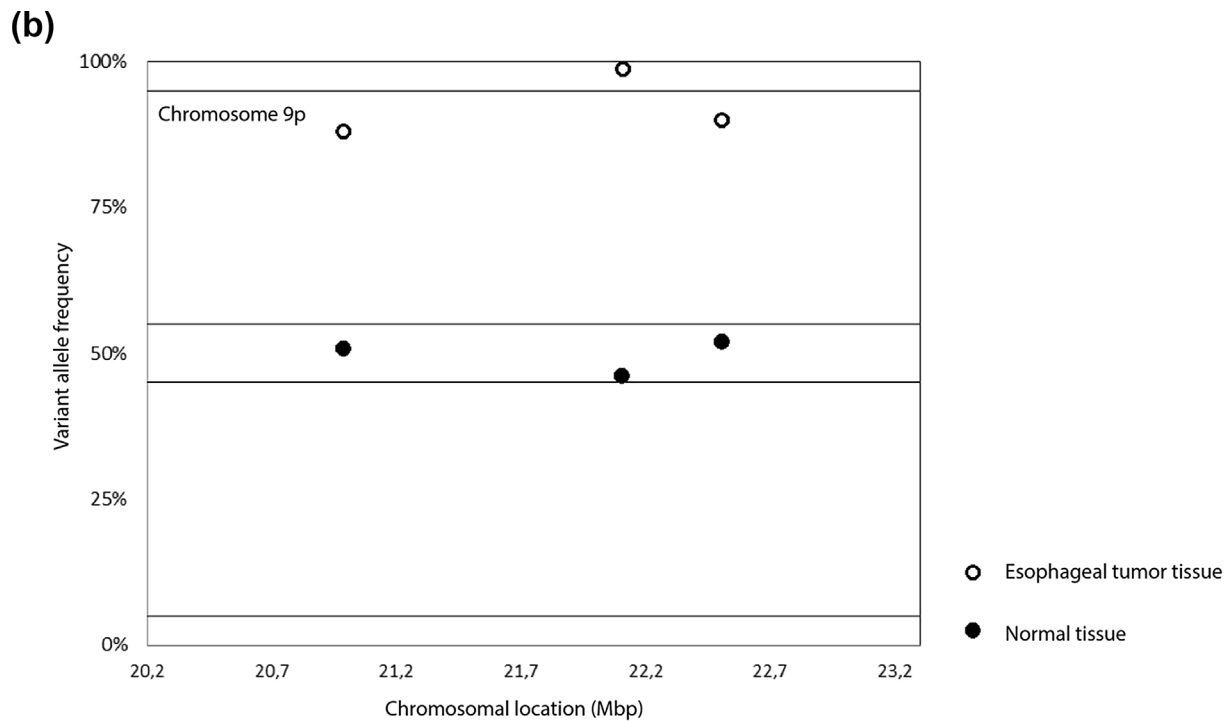


Fig. 4b. NGS-based variant allele frequency via SNP analysis in the first patient. The X-axis shows the location on chromosome 9p in million base pairs (Mbp). The Y-axis shows the percentage of variant allele frequency. LOH was unambiguously demonstrated on several locations of chromosome 9p near the *CDKN2A* locus of the first FAMMM patient.

Hence, these results strongly suggest that ESCC developed in the context of FAMMM syndrome in both patients.

Pathogenic *CDKN2A* germline variants are associated with increased risk for developing melanomas and pancreatic cancer. A study with 19 families with a pathogenic *CDKN2A* germline variant showed a wide diversity in cancer presentation, including three patients with esophageal cancer. One had small cell carcinoma and two a malignancy

of unknown histology (Lynch et al., 2002). Another recent study also reported that FAMMM patients with a pathogenic *CDKN2A* variant are at increased risk for the early onset of several malignancies (Middlebrooks et al., 2019). It was suggested that, besides melanomas and pancreatic cancer, also a higher proportion of malignancies in the colon, nervous system, soft tissue, bone, testis, ovary and esophagus was present in mutation carriers versus non-carriers.

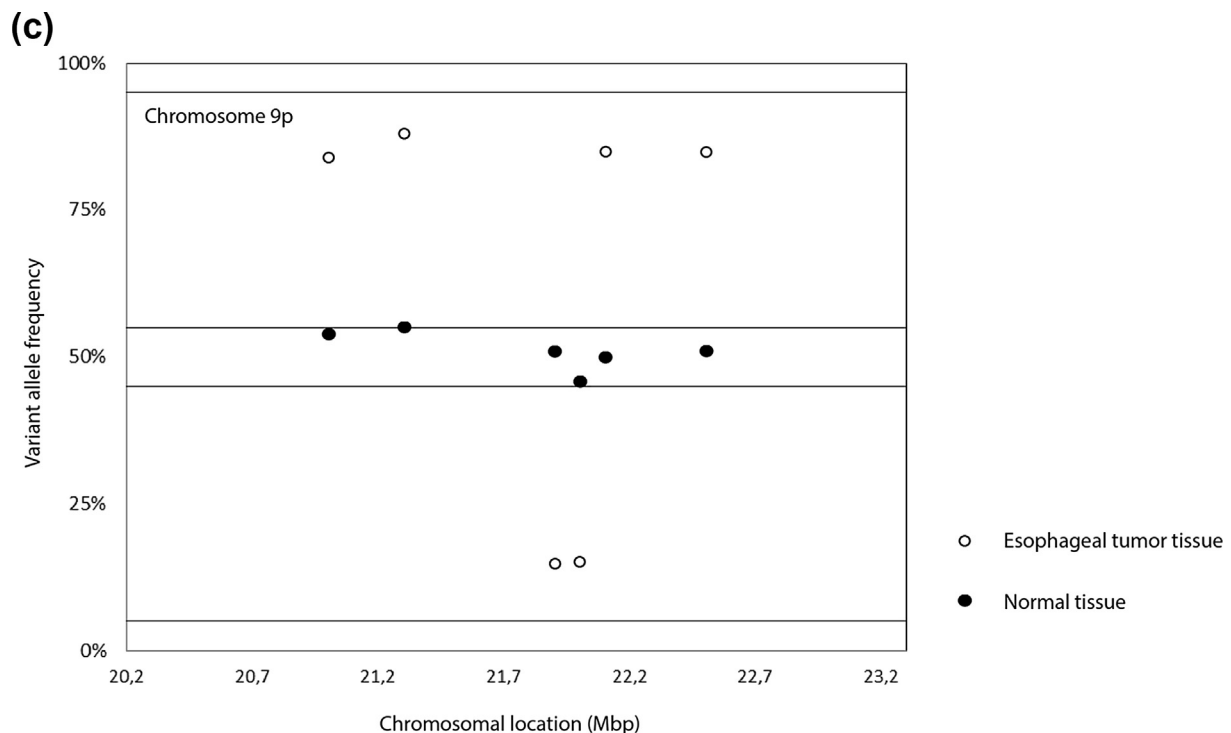


Fig. 4c. Also in the second patient, LOH was unambiguously demonstrated on several locations of chromosome 9p both near and in the *CDKN2A* locus.

CDKN2A mutations are reported in precancerous esophageal lesions and the mutations can thus be an early event in the ESCC carcinogenesis (Liu et al., 2017). Additionally, somatic inactivation of the *CDKN2A* gene is frequently reported in sporadic ESCC patients, mostly via mutations or epigenetic processes like promotor hypermethylation (Abbaszadegan et al., 2005; N. Cancer Genome Atlas Research et al., 2017). Several studies reported somatic (epigenetic) silencing of *CDKN2A* in ESCC ranging from 25% to 76%; one of these studies found biallelic inactivation in 13% of patients with ESCC (Hu et al., 2004). This indicates that patients with a germline pathogenic *CDKN2A* variant may be at higher risk of ESCC, but further studies are needed to clarify the prevalence and clinical relevance of the combination.

Since tobacco use was reported to increase the risk for development of several (squamous cell) cancers in FAMMM patients, one could not rule out this had a synergistic effect for development of the tumor in one of our patients (Potjer et al., 2015). This underlines the need to discourage carriers of a pathogenic *CDKN2A* variant not to smoke. Not only to prevent lung, pancreatic and head and neck cancer, but also to prevent esophageal cancer.

In conclusion, this study suggests that ESCC developed in the context of FAMMM syndrome in the two presented patients and a relation between *CDKN2A* and esophageal cancer is also supported by the currently available literature. Patients with a pathogenic germline variant in *CDKN2A* may be at higher risk for developing ESCC and alertness for clinical features of esophageal cancer should be warranted in these patients. However, since earlier reports on ESCC with FAMMM are scarce, future studies are needed to clarify both the prevalence and the clinical relevance of this combination.

CRediT authorship contribution statement

Berend J. van der Wilk: Conceptualization, Writing - original draft, Project administration, Visualization. **Bo J. Noordman:** Conceptualization, Writing - review & editing. **Peggy N. Atmodimedjo:** Conceptualization, Writing - review & editing, Methodology, Formal analysis. **Winand N.M. Dinjens:** Conceptualization, Writing - review & editing, Methodology, Formal analysis. **Robert J.F. Laheij:** Conceptualization, Writing - review & editing, Resources. **Anja Wagner:** Conceptualization, Writing - review & editing. **Bas P.L. Wijnhoven:** Conceptualization, Writing - review & editing, Resources. **J. Jan B. van Lanschoot:** Conceptualization, Writing - review & editing, Supervision, Resources.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmg.2020.103840>.

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