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Two co-existing germline mutations *P53 V157D* and *PMS2 R20Q* promote tumorigenesis in a familial cancer syndrome

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Abstract

Germline mutations are responsible for familial cancer syndromes which account for approximately 5–10% of all types of cancers. These mutations mainly occur at tumor suppressor genes or genome stability genes, such as DNA repair genes. Here we have identified a cancer predisposition family, in which eight members were inflicted with a wide spectrum of cancer including one diagnosed with lung cancer at 22 years old. Sequencing analysis of tumor samples as well as histologically normal specimens identified two germline mutations co-existing in the familial cancer syndrome, the mutation of tumor suppressor gene *P53 V157D* and mismatch repair gene *PMS2 R20Q*. We further demonstrate that *P53 V157D* and/or *PMS2 R20Q* mutant promotes lung cancer cell proliferation. These two mutants are capable of promoting colony formation in soft agar as well as tumor formation in transgenic drosophila system. Collectively, these data have uncovered the important role of co-existing germline *P53* and *PMS2* mutations in the familial cancer syndrome development.

Keywords

P53 V157D; *PMS2 R20Q*; Germline mutation; Familial cancer syndrome; Co-existing

1. Introduction

Cancer is commonly considered as an aging related disease, with the increasing risk of various accumulated mutations at tumor suppressor genes, genome stability genes, such as DNA repair genes, as well as oncogenes [1]. Familial cancer, accounting for approximately

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Conflict of Interest

The authors declare no conflict of interests.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.canlet.2013.08.032>.

5–10% of all types of cancers [2], usually happens in the early stage of one's life, presumably due to the constitutive and continuous action of the cancer-predisposition genetic alterations.

Familiar cancer syndrome frequently caused by genetic alterations occurring at either tumor suppressor genes or genome stability genes, such as DNA repair genes. The most famous familiar cancer syndrome Li–Fraumeni syndrome (LFS), which is characterized by an inheritable sarcoma before 45 years old, is dominantly caused by germline mutations of tumor suppressor gene *P53*. Moreover, approximately 30% of the Li–Fraumeni-like syndrome families, which predisposed to LFS but did not precisely meet the classic diagnostic criteria including onset age and tumor types, harbor *P53* mutations [2]. Genome stability genes, such as DNA mismatch repair genes, is another type of genes frequently mutated in cancer predisposition family [3]. DNA mismatch repair is a highly conserved DNA repair pathway in the correction of base-pair mismatches generated during DNA replication and recombination. In eukaryotes, the first step of mismatch repair is the recognition of mismatches by a heterodimer of *MSH2* and *MSH6* [4,5]. A heterodimer of *MLH1* and *PMS2*, directs excision of the newly synthesized, error-containing strand and subsequent gap repair by DNA polymerase and DNA ligase [6]. In addition, *PMS2* has been shown to be necessary for cisplatin-induced activation of *P73*, a transcription factor of the *P53* family with proapoptotic activity [7]. Mutation of these genes may de-regulate the activity of DNA mismatch repair and thus contribute to DNA mutagenesis and ensued tumorigenesis [8–10].

Here we have investigated a lung cancer patient who got an early onset of lung tumorigenesis at the age of twenty-two, from which we have identified a family with hereditary cancer syndrome potentially associated with two co-existing germline mutations including the tumor suppressor gene *P53* mutation and the mismatch repair gene *PMS2* mutation. Through functional biology assays *in vitro* and *in vivo* transgenic drosophila system, we uncover an important role of the tumor suppressor gene *P53* mutation and mismatch repair gene *PMS2* mutation in promoting tumorigenesis.

2. Materials and methods

2.1. Patients and diagnosis

A 22-year-old young man with a three-week history of post-exercise chest pain at right thorax was admitted to Shanghai Cancer Hospital, Fudan University, in Shanghai, China. He then received a comprehensive health examination and chest X-ray scanning and enhanced lung CT scanning. After diagnosis of lung cancer, he received surgical operation after signing the patient consent.

2.2. Clinical samples

Clinical sample collection in this study was approved by the local ethics committees. Both lung tumor and histologically normal lung tissue from distant area were dissected and snap frozen instantly after surgery. The colon cancer specimen from the proband's father was provided in the form of archived H&E stained slides prepared about 20 years ago in Xu Zhou People's Hospital, Jiangsu Province. Histologically normal lymph node in H&E stained slide were used as normal control. Blood samples from the proband's families are collected with individual consents.

2.3. RNA and DNA preparation

RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. RNA samples were reverse transcribed using RevertAid First

Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA) as previously described [11]. Genomic DNA for lung cancer sample, distant normal lung tissue and blood samples were extracted using Gentra Puregene Tissue Kit (Qiagen, Hamburg, Germany). Genomic DNA from colon cancer and lymph node were extracted using QIAamp DNA FFPE Tissue Kit (Qiagen) following the standard protocol.

2.4. PCR and sequencing

EGFR and *KRAS* mutation detection were previously described [11]. PCR product amplified from cDNA was directly sequenced after gel purification. All mutations found at cDNA level were confirmed at genomic DNA. For *PMS2* genomic DNA sequencing, due to many pseudogenes in the genome, long-range PCR was used for the lung cancer, distant normal lung and blood genomic DNA [12]. Candidate genes previously reported in familial cancer syndromes, including tumor suppressor genes like *P53*, *PTEN*, and DNA mismatch repair genes like *PMS2*, *MSH2*, *MLH1* and *MSH6* were sequenced and analyzed for mutational status. Primers for PCR amplification and regular sequencing were list in Supplementary Tables 1 and 2. Considering the low amount and poor quality of long-term archived clinical samples, *PMS2* mutation detection in colon cancer and corresponding lymph node from the proband's father was carried out by nested PCR using genomic DNA, and the PCR products were cloned into T-vector and transformed into DH5α.

2.5. Plasmids and viral infection

WT and mutated *P53* and *PMS2* were cloned into lenti-virus based vector pCDH-CMV-MCS-EF1-copGFP using *XbaI* and *NotI*. Viruses were packaged using HEK293T cells cultured with DMEM plus 10% FBS as described previously [13].

2.6. Foci formation assay

Mouse embryonic fibroblasts (MEFs) derived from *loxp-stop-loxp-Kras*^{G12D} mice were prepared as previously described and were genotyped by *Kras*^{G12D} primers [14]. The MEFs was then treated with Adeno-Cre (8×10^6 pfu) to induce the expression of *Kras*^{G12D}. MEFs were then used for viral infection for expression of either *P53* V157D or *PMS2* R20R or both and then culture for 3 weeks until foci formation. The MEFs was then fixed with 4% PFA and stained with crystal violet for foci counting as previously described [15].

2.7. Soft agar assay

For anchorage-independent cell growth, cells were plated in 0.4% agarose on top of a 1% agarose base supplemented with complete medium. Cells were maintained for about 4 weeks before staining with 0.005% crystal violet. The pictures were taken by digital camera or microscope and the number of colonies were counted by Quantity One software (Bio-Rad, Hercules, CA, USA).

2.8. Real-time PCR and western blot

Total RNA was prepared and retro-transcribed into first-strand cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The cDNAs were then used for real-time PCR on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR-Green Master PCR mix (Toyobo, Osaka, Japan). *β-actin* served as internal control. Primers used for RT-PCR were listed as following: For mouse *p21*, forward primer: 5'-CGAGAACGGTGGAACTTTGAC-3'; reverse primer: 5'-CAGGGCTCAGGTAGACCTTG-3'. For *β-actin*, forward primer: 5'-cgttgacatccgtaagacc-3'; reverse primer: 5'-aacagtcgcgctagaagcac-3'. Total protein lysate were prepared by homogenization in protein loading buffer. Equal amounts of protein from each sample were separated by electrophoresis on an SDS-PAGE gel and transferred onto

PVDF membranes. Western blot analysis was performed using following antibodies towards: P53 (Epitomics, Burlingame, CA, USA), PMS2 (Bio-world Technology, Newmarket, Suffolk, UK) and β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.9. Transgenic drosophila

To construct *UAS-P53 WT*, *UAS-PMS2 WT*, *UAS-P53 V157D*, *UAS-PMS2 R20Q*, cDNAs were amplified by RT-PCR from the patient's tissues. Transgenic fly lines were generated using pUAST vector by standard P-element mediated transformation and were as follows: *UAS-P53 WT*, *UAS-PMS2 WT*, *UAS-P53 V157D*, *UAS-PMS2 R20Q*, *UAS-P53 V157D + PMS2 R20Q*. GMR was a GAL4 strain to drive the transgenes expression in fly eyes. GMR-Gal4 was described before [16]. Flies were crossed and raised at 25 °C. Standard methods were used to recombine and balance chromosomes bearing mutations and transgenes.

2.10. Larva and pupal UV irradiation assay

For pupal UV irradiation assay, pupae (24 h after puparium formation) were collected and the pupal case was removed from around the retina area just before irradiation. The pupae were immobilized on the side so that only one retina (right side) was exposed to UV. All irradiations were performed using a 20W 254 nm UV light at a distance of 44 cm. After irradiation, pupae were kept in the dark until being processed [17,18].

2.11. Immunostaining of imaginal discs

Immunostaining was performed following standard protocols. Eye discs of 3rd-instar larva were fixed in 1 × PBS plus 4% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 20 min at room temperature. Samples were washed in PBST (1 × PBS, 0.1% Triton X-100), and incubated with primary antibody cleaved caspase-3 (Cell Signaling Technology) 1:50 overnight at 4 °C and secondary antibody for 2 h at room temperature diluted in PBST. Then the positive cleaved caspase-3 staining in per eye disc were counted and statistical analysis was carried out.

3. Results

3.1. Identification of a 22-year-old young man with EGFR mutant lung adenocarcinoma

A 22-year-old man was admitted to Shanghai Cancer hospital with a mass of a diameter approximately 4 cm in the upper lobe of right lung according to chest X-ray scanning (Fig. 1A, left panel, arrow). No obvious lesion was found in the left lung. Enhanced lung CT scanning indicated that the mass is about 4.8 cm by 5.2 cm with clear margin (Fig. 1A, right panel, arrow). No meta-static lesion in brain and other organs was found (data not shown). Pathological analysis of the dissected lesion had confirmed that the patient had a moderately differentiated lung adenocarcinoma (Fig. 1B). The tumor was non-invasive to visceral pleura. However, cancer metastasis was observed in most draining lymph nodes. Through direct sequencing of PCR product from genomic DNA, we found an *EGFR* 746–750 ELREA deletion in the lung tumor but not in pathologically normal lung tissue from distant area (Fig. 1C).

3.2. Identification of a cancer predisposition family

The unusual early onset of lung tumorigenesis in this patient triggered our interest for further analysis of his family history. Strikingly, there were eight direct members in his family died from a variety of cancers including colorectal cancer, liver cancer, breast cancer, lung cancer as well as leukemia (Fig. 2). All of them were diagnosed with a variety of cancer types under 54 years old while about half of them developed cancers rather early, at

less than 31 years old (Fig. 2). These data indicate the existence of a familial cancer predisposition syndrome in this family. We hereby refer the young patient to the proband.

3.3. Germline mutations *P53* V157D and *PMS2* R20Q co-exist in the proband's lung tumor

We then asked what genetic alteration is potentially involved in this familial cancer-prone syndrome. Several candidate genes have been previously reported to be involved in familial cancer syndrome, including tumor suppressor genes such as *P53*, *PTEN*, and DNA mismatch repair genes such as *PMS2*, *MSH2*, *MLH1* and *MSH6* [3]. Thus, we analyzed all these genes via direct PCR sequencing using cDNA derived from lung tumor and paired pathologically normal lung tissue from distant area of the proband. Interestingly, we found that the proband carried *P53* V157D mutation in his distant normal lung (Fig. 3A), suggesting that the *P53* germline mutation predisposes the cancer-prone syndrome. More strikingly, in addition to the *P53* germline mutation, a *PMS2* point mutation R20Q was found in normal lung from the proband (Fig. 3B). However, there is no mutation was observed from any other genes we have analyzed (Supplementary Table 3).

3.4. Germline mutations *P53* V157D and *PMS2* R20Q present in the proband's father

To confirm the germline *P53* and *PMS2* mutations, we first obtained the blood samples from the proband's mother and elder sister who are perfectly healthy. No mutation of either *P53* V157D or *PMS2* R20Q was found in their genomic DNA (Supplementary Fig. S1). Since the proband's father died of colon cancer about two decades ago, we could only archived the hematoxylin and eosin-stained slides for his colon cancer and the paired pathologically normal lymph node specimens (Fig. 4A and data not shown). We then performed the genomic DNA analysis on these samples through micro-dissection. A *Kras*^{G12D} mutation was found in colon cancer specimens but not the corresponding lymph node specimen (Fig. 4B and data not shown). Interestingly, we found the exact same *P53* V157D mutation in both colon tumor and lymph node specimens from the proband's father (Fig. 4C).

To test whether *P53* V157D mutation potentially contributes to the familial cancer syndrome, we first detected the transcriptional regulation of *p21* which is a target of p53 and inhibitor of cyclindependent kinases and cell cycle progression [19,20]. Our data showed that *P53* V157D mutation failed to transactivate *p21* expression in *P53*^{-/-}, *K-ras*^{G12D} mice embryonic fibroblasts (Supplementary Fig. S2). Accordingly, in contrast to WT *P53*, no obvious growth inhibition was observed in *P53* V157D-overexpressed *P53*^{-/-}, *K-ras*^{G12D} MEFs (Supplementary Fig. S2), suggesting that *P53* V157D mutation may lead to uncontrolled proliferation in *P53*^{-/-}, *K-ras*^{G12D} MEFs and contributes to the familial cancer syndrome.

Due to the finding of concurrent germline mutations of *P53* and *PMS2* in the proband, we are interested to investigate the *PMS2* mutational status in the proband's father. Since the archived samples were prepared more than 20 years ago, the quality of RNA is insufficient for *PMS2* mutation analysis as we have done for the proband. Therefore, we tried genomic DNA for further genetic analysis. Giving the fact that human genome contains 12 pseudogenes of *PMS2* [12], we cloned the nested PCR products of *PMS2* into T-vector and screened clones using allele-specific primers as previously described [21]. We first tested the methods in the proband's genomic DNA and found that 6 out of 48 clones were mutant for *PMS2* in his normal lung sample and 7 out of 47 clones were mutant for *PMS2* in his lung cancer sample (Supplementary Table 4). A comparable ratio of *PMS2* mutation (4/46) was also seen in the normal lymph node specimen from the proband's father, indicating the germline origin for *PMS2* mutation (Fig. 4D). Interestingly, the colon cancer sample showed an even higher ratio for *PMS2* mutation (28/47) (Fig. 4D), which implies a potential loss of heterozygosity during the proband father's tumor formation process. Taken together, the

coexistence of both *P53 V157D* and *PMS2 R20Q* germline mutations in the proband as well as his father may give an explanation for this familial cancer syndrome.

3.5. *P53 V157D* and/or *PMS2 R20Q* promote tumor formation

We next expressed *P53 V157D* and/or *PMS2 R20Q* in lung cancer cell lines to test the potential role of *P53 V157D* and/or *PMS2 R20Q* in promoting tumorigenesis. Interestingly, *P53 V157D* and/or *PMS2 R20Q* could promote cell proliferation in A549 (*P53* wild-type) and CRL-5872 (*P53* mutant) cells (Fig. 5A and B; Supplementary Fig. S3A and B). We also observe that co-expression of *P53 V157D* and *PMS2 R20Q* enhanced the colony-forming ability of the A549 and CRL-5872 in soft agar assay (Fig. 5C and D; Supplementary Fig. S3C and D). The cooperative role of *P53 V157D* and *PMS2 R20Q* in tumor formation was also found in the *P53*^{-/-}, *K-ras*^{G12D} mice embryonic fibroblasts (Supplementary Fig. S4A and B). Moreover, we observed that co-expression of *P53 V157D* and *PMS2 R20Q* increased the foci formation ability of *K-ras*^{G12D} mice embryonic fibroblasts (Supplementary Fig. S4C). Taken together, these results show that the *P53 V157D* and/or *PMS2 R20Q* could promote tumor formation *in vitro*.

We next checked the role of *P53* and/or *PMS2* mutants in tumorigenesis using *in vivo* transgenic drosophila system. The drosophila is an excellent model to study gene function. Since the eye of the drosophila is superficial and accessible and highly convenient to observe, we use the GMR drosophila which has a GAL4 strain to drive the trans-genes expression in fly eyes to confirm our hypothesis. We first constructed the *P53 V157D* and/or *PMS2 R20Q* transgenic drosophila and generated different groups of transgenic flies including *P53 WT*, *PMS2 WT*, *P53 V157D*, *PMS2 R20Q*, *P53 V157D/ PMS2 R20Q*. Unexpectedly, all the flies showed normal pathology and no tumor were observed (Fig. 6A).

Since both *P53* and *PMS2* are important for maintaining genome stability, we reasoned that additional DNA damage may provide a good system for testing the function of these two mutants. Pupae collected 24 h after puparium formation were exposed to UV irradiation only on the retina from right side with the pupal case removed as previously described [17,18]. Interestingly, in contrast to normal growth pattern in control flies (Supplementary Fig. S5A), we did observe a clear tumor formation in the *P53 V157D* and/or *PMS2 R20Q* transgenic flies (Fig. 6B and Supplementary Fig. S5B). The incidence of the tumor formation was almost 100% in these *P53 V157D/PMS2 R20Q* bitransgenic flies while only about 50–60% of either *P53 V157D* or *PMS2 R20Q* single transgenic flies developed tumor in eyes. The WT *P53* transgenic flies showed a shrunk size of eye, indicating a role of WT *P53* in inhibiting cell growth and proliferation. We further analyzed the cleaved caspase-3 level in the eye disc at the earlier period after UV irradiation. Immunofluorescence results showed that expression of either *P53 V157D* or *PMS2 R20Q* significantly protected the cells from apoptosis in contrast to normal controls. An even lower rate of apoptosis were observed in the case of *P53 V157D/PMS2 R20Q* bitransgenic flies (Fig. 6C and D; Supplementary Fig. S6). Taken together, these data have demonstrated that the germline mutants *P53 V157D* and *PMS2 R20Q* are capable of promoting tumor formation *in vitro* and *in vivo*.

4. Discussion

In this study, we have identified a family with eight direct members inflicted with a variety type of cancer by tracing a young lung adenocarcinoma patient with *EGFR* kinase domain mutation. Interestingly, we have found that the previously unreported *P53 V157D* and *PMS2 R20Q* germline mutations are potentially responsible for cancer predisposition syndrome in

this family. Using *in vitro* and *in vivo* biological function assays, we have displayed that these germline mutants could facilitate the tumor formation process.

Our findings here suggest that the germline mutants *P53 V157D* and *PMS2 R20Q* play an important role in the family inflicted by a cancer predisposition syndrome. This is different from the previously reported Li–Fraumeni-Like (LFL) syndrome, the occurrence of which is frequently due to the germline mutation of a single tumor suppressor gene, e.g., *P53* [22–24]. Previous studies have reported that *PMS2* can interact with *P53*-related transcription factor *P73*, which is required for the stabilization and the proapoptotic function of *P73* [25]. Besides, the apoptotic function of human *PMS2* was compromised by the single-nucleotide polymorphic variant *R20Q* [26]. Thus, the coexistence of *P53* and *PMS2* germline mutations may lead to lack of DNA damage-induced apoptosis and partially explain the early onset of the lung adenocarcinoma development. Our study has further revealed the tumor-promotive function of these two mutants *P53 V157D* and *PMS2 R20Q* from *in vitro* studies. However, there is no tumor observed in the transgenic flies with ectopic expression of *P53 V157D* and/or *PMS2 R20Q*. We reason that the *in vivo* function of these two mutants might be prominent under the DNA damage condition since both genes are important for maintaining the genome stability. This turns out to be true and we have actually observed that ectopic expression of either mutant promotes the tumor formation in the eyes of drosophila after UV irradiation. The tumor-promotive effect is observed in the case of the bitransgenic flies with expression of both *PMS2 R20Q* and *P53 V157D*. However, the potential functional interaction of these two mutants is not clear and definitely requires future studies.

In summary, we have identified a familiar syndrome with the germline mutations of both tumor suppressor gene *P53* and mismatch repair gene *PMS2*. Both mutants are capable of promoting tumor formation from *in vitro* study as well as *in vivo* model system study under the DNA-damaging condition. These data may provide a novel insight into the cancer development mechanism via the potential functional interaction between the loss-of-function mutants of a tumor suppressor and a DNA mismatch repair gene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Identification of a 22-year-old young man with lung adenocarcinoma positive for EGFR kinase domain mutation. (A) Chest X-ray scanning of the young patient (Left panel). Enhanced CT was carried out and the results indicated that the mass is about 4.8 cm by 5.2 cm with clear margin (Right panel). (B) Pathological analysis of the lung tumor (Scale bar: 100 μm). (C) *EGFR* exon 19 deletion 746–750 ELREA presents in the lung tumor but not in the pathologically normal lung tissue from distant area.

Fig. 2. Identification of a cancer predisposition family. Eight direct family members were inflicted by a variety of cancers including colorectal cancer, liver cancer, breast cancer, lung cancer as well as leukemia.

Fig. 3. Coexistence of *P53* V157D and *PMS2* R20Q germline mutations in the proband. (A) The proband harbors *P53* V157D mutation in his normal lung tissue as well as his lung cancer samples. (B) *PMS2* R20Q mutation was found in both lung tumor and paired distant normal lung tissue from the proband.

Fig. 4. Coexistence of *P53* V157D and *PMS2* R20Q germline mutations in the proband's father. (A) Histology of colon cancer specimens from the proband's father. Scale bar: 100 μ m. (B) *K-RAS*^{G12D} mutation was found in colon cancer sample. (C) Heterozygous *P53* V157D mutation was found in both colon tumor and paired pathologically normal lymph node from the proband's father. (D) Clone screening showed that 28 out of 47 clones from the colon tumor and 4 out of 46 clones from the pathologically normal lymph node were *PMS2* R20Q mutants.

Fig. 5. The function of *P53 V157D* and/or *PMS2 R20Q* mutants in promoting tumorigenesis *in vitro*. (A) Ectopic expression of *P53 V157D* and/or *PMS2 R20Q* in A549 cells. (B) *P53 V157D* and/or *PMS2 R20Q* promoted cell proliferation in A549 cells. (C) *P53 V157D* and/or *PMS2 R20Q* in A549 cells promoted colony formation in soft agar assay. Scale bar: 100 μ m. (D) Statistical analysis of colony formation of A549 cells in soft agar. #: these colonies grew up as GFP negative, indicating of no WT *P53* expression. Data were shown as mean $\pm$ s.e.m. * $P < 0.05$.

Fig. 6. *P53 V157D* and/or *PMS2 R20Q* mutants promotes tumorigenesis in transgenic flies after UV irradiation. (A) The *P53 V157D* and/or *PMS2 R20Q* transgenic adult flies showed normal cell growth in eyes in comparison with normal control. (B) *P53 V157D* and/or *PMS2 R20Q* significantly increased the tumor formation in the adult fly eye after UV treatment while the *P53 WT* and *PMS2 WT* decreased the eye size in comparison with normal control. The tumor incidence is indicated at the lower right corner. (C) *P53 V157D* mutant and/or *PMS2 R20Q* mutant significantly decreased the cell apoptosis in eye disc with UV irradiation in comparison with normal control. (D) Statistical analysis of caspase-3-positive staining in C. Data were shown as mean $\pm$ s.e.m.. ** $P < 0.01$.