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THE LI FRAUMENI SYNDROME (LFS): A MODEL FOR THE INITIATION OF P53 SIGNATURES IN THE DISTAL FALLOPIAN TUBE

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Abstract

A candidate early precursor to pelvic serous cancer – the “p53 signature” – is commonly found in the benign mucosa of the distal fallopian tube and harbors *p53* mutations and evidence of DNA damage. We examined tubes from women with pre-existing (germ-line) mutations in *p53* (Li Fraumeni Syndrome or LFS) for evidence of this precursor. Fallopian tubes from two cases of LFS were immunostained for p53, Ki-67 (proliferation) and H2AX (DNA damage response) and analyzed for *p53* mutations by laser capture micro-dissection (LCM) and *p53* genomic sequencing (exons 2-11). A common single nucleotide repeat (snp) in exon 3 (rs 1042522) and deletion sequencing chromatograms in exon 4 were examined in combination to estimate LOH in both LFS tubes and advanced serous carcinomas from the general population. LFS tubal epithelium contained abundant (10-20 per section) p53 signatures with evidence of DNA damage and low proliferative activity. Six of 11 LFS microdissected p53 signatures (55%) and 15 of 21 serous carcinomas (71%) revealed LOH at *p53* locus relative to background epithelium. The LFS model confirms prior observations that the distal fallopian tube is particularly prone to focal epithelial *p53* gene inactivation – *p53* mutation and LOH- in the absence of malignancy or increased epithelial proliferation. The fact that the LFS is not associated with ovarian cancers is consistent with the concept that loss of p53 function must be accompanied by at least one more genotoxic event (BRCA1/2 functional inactivation) to produce the malignant phenotype. This is in keeping

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with a general model of carcinogenesis, in which different and often independent risk factors operate at multiple points in the serous carcinogenic spectrum.

Introduction

Pelvic serous carcinoma (“ovarian cancer”) typically presents in an advanced stage, with a brief interval from the onset of symptoms to the discovery of widespread disease. Because of its rapid evolution and propensity for growth on ovarian and peritoneal surfaces, serous cancer was presumed by many to originate from the ovarian surface epithelium or OSE.¹ However, serous ovarian cancers are rarely identified early in their development and classifications of precursor lesions in the OSE have not been uniform.^{2 3 4 5} Recent studies on the pathogenesis of serous ovarian carcinoma have highlighted the distal fallopian tube as a potential site of origin for many of these pelvic tumors. Several authors have documented non invasive (intraepithelial) serous carcinomas in the distal tubal mucosa of prophylactic salpingectomies of women with BRCA1 or BRCA2 mutations (BRCA+), as well as women with presumed ovarian and peritoneal serous carcinomas.^{6 7 8 9 10 11 12} Coupled with prior observations in the fallopian tube, these findings have raised the possibility that a serous carcinogenic sequence exists in the distal fallopian tube, and have generated a new paradigm for both early detection and prevention of ovarian cancer.¹³ Moreover, a potential non-malignant precursor to serous carcinoma – termed the p53-signature – has been recently described.¹⁴ This entity possesses several characteristics in common with serous carcinomas of the fallopian tube, including: 1) typical location in the distal fallopian tube (fimbria), 2) intense p53 immunostaining, 3) the secretory cell phenotype (BCL-2 and HMFG2+), 4) a DNA damage response signified by immunohistochemical localization of H2AX, 5) presence of ovarian cancer-related p53 mutations, 6) physical proximity to and shared p53 mutations with carcinoma , and 7) similar epidemiologic risk factors including low parity and low body mass index.^{14 15 16 17}

Although the p53 signatures is occasionally seen in continuity with early (intraepithelial) serous carcinoma of the tube, they are typically found in normal appearing epithelium of the tubal fimbria and the sequence of genetic events leading to their formation and their progression to malignancy is unclear.^{12 14} An important variable in their development is the distal fallopian tube but even in this site, the number of p53 signatures rarely exceeds 2-3 per case.

Li Fraumeni syndrome (LFS) is a rare familial disorder characterized by an inherited (germ-line) mutation in p53. Individuals with LFS appear prone to a range of malignancies at a rate estimated as high as 50-fold over the general population, which may be further increased by exposure to ionizing radiation.^{18 19} These patients are at increased risk for breast cancer, but the link between ovarian cancer and LFS is not established. Nevertheless, as LFS is characterized by germ-line p53 mutations, we explored the possibility that this variable would influence the risk of development of p53 signatures in the distal fallopian tubes of these individuals.

Materials and methods

Case material

This study was approved by the Institutional Review Board at Brigham and Women's Hospital. Two patients with LFS from which normal appearing fallopian tubes had been removed during surgical procedures were identified and slides retrieved from the pathology files at Brigham and Women's Hospital and Massachusetts General Hospital. Additional archived pelvic serous carcinomas in the files at Brigham and Women's Hospital were

included. The tissues analyzed included the following: 1) fallopian tubes from two cases of LFS; 2) 21 serous ovarian and tubal carcinomas from the general population. Frequencies of p53 signatures identified in (1) were compared to those for other populations described previously.¹⁴⁻²⁰

Immunophenotyping

Sections from the LFS tubes were analyzed for a marker of proliferation (MIB-1 corresponding to Ki-67; M7240, DAKO, Carpinteria, CA) and p53 (OP43, Oncogene Science, Cambridge, MA), each at 1:100 dilution. Nuclear MIB-1 staining index was estimated as a function of the number of p53 positive cells in a given field.¹⁴

H2AX was used as a marker for DNA damage. Punctuate staining by this marker is associated with γ -H2AX, the phosphorylated form of the core histone γ -H2AX that localizes to the vicinity of - and is recognized as a marker for - double-stranded DNA breakage (Upstate Cell Signaling Solution, Charlottesville, VA; monoclonal JBW301, 1:200). H2AX is phosphorylated at a unique serine residue (S139) in its C-terminus at sites of DNA double strand breaks.¹⁶ This staining was only performed on the fallopian tubes from the patients with LFS.

An antibody to bcl-2, an inhibitor of apoptosis, which has been demonstrated to selectively highlight the cytoplasm of secretory epithelial cells in the fallopian tube, was also used.²¹

Antigen-antibody complexes were localized with the Envision technique using peroxidase and diaminobenzidine as the reporter, as previously described.¹⁴

P53 mutation analysis

Selected tissue blocks containing p53 signature-positive and negative loci from patients with LFS were serially sectioned and the preservation of the p53 epithelial signature confirmed by immunostaining. Laser capture microdissection (LCM) was used to isolate p53 signature and control somatic DNA from selected cases using the PALM microbeam instrument. Samples were incubated at 62 degrees C. with proteinase K for 48 hours followed by the addition of Chelex (Biorad, Burlingame, CA) and boiling for 10 minutes. Genomic DNA was amplified by polymerase chain reaction (PCR), using tailed primers designed to amplify exons 2, 3, 5-9 and 11 of p53. A secondary amplification was performed using primers specific to the tail sequence used in the primary amplification. PCR products were then sequenced from both strands using tail primers. Data was analyzed using the Mutation Surveyor program (Soft Genetics, State College, PA). Candidate mutations found by the software were compared to a reference database for cancer-associated p53 mutations (Universal mutation database, <http://www.umd.be:2072/IFAMTP53A.shtml>).

To assess possible LOH for the p53 locus, sequencing chromatograms in exon 5 containing the patient specific LFS mutation were examined. Allele loss at a heterozygous common single nucleotide repeat (snp) in exon 4 (rs1042522) was also examined to determine LOH, and the two results were combined to estimate heterozygosity.

Results

Fallopian tubes from women with LFS display a markedly increased frequency of p53 positive epithelia, particularly in the distal fallopian tube

(Figures 1A and 1B). Numerous linear segments of p53 positive epithelium were identified, including as many as 20 in each section of the fimbria. The frequency of p53 positive epithelium in the more proximal tube was reduced relative to the fimbria but was elevated in

all sections. In the endometrium of LFS women, p53 was negative in the epithelia with the exception of a single normal appearing gland in one section (not shown).

In contrast to the p53 positive epithelia, the adjacent epithelium demonstrated variable but weaker p53 staining (Figure 1 A&B) confined to the secretory cells. This pattern of immunostaining is similar normal mucosa of the general population (not shown). In addition, the number of p53 positive nuclei in the LFS tubes varied, with numerous foci containing less than 12 consecutive p53 positive nuclei, including many single cells, indicating p53 positive cells that apparently had not undergone cell division following the acquisition of p53 positivity. Overall, the proliferative index (MIB-1) was low (Figure 1C).

P53 positive foci in fimbria of the patients with LFS exhibit similar DNA damage response to p53 signatures (Figure 2)

Figure 2B depicts a p53 positive focus of cells immunostained with H2AX (Figure 2C).¹⁴ Small numbers of nuclei contained punctuate immunopositivity consistent with intra-nuclear localization of γ -H2AX (arrows), similar to prior descriptions of p53 signatures in other populations.¹⁴ Single, p53 positive cells also contained evidence of DNA damage via H2AX staining (not shown).

P53 positive foci in LFS are identical in cell type to p53 signatures

Immunostains for bcl-2 revealed strong cytoplasmic staining of the p53-H2AX signatures in LFS, consistent with their origin in secretory epithelial cells (Figures 2D and 2E). The generally low proliferative index of these p53 positive foci was similar to other studies of p53 signatures (Figure 1C).^{13 14 20}

P53 signatures associated with LFS and pelvic serous carcinomas display high frequencies of LOH at a second p53 allele

P53 sequence analysis from all 11 p53-H2AX signatures revealed a single germ-line mutation (482_487delCCATCT). Further sequence analysis designed to identify LOH revealed the following; heterozygosity in 5 and loss of the wild type allele in 6 relative to the control tissue, for a frequency of 55% (Table 1 and Figure 3). Of 21 consecutive pelvic serous carcinomas analyzed, 15 (71%) harbored both a point mutation and evidence of LOH (Table 1). The difference in frequencies of LOH in the two groups was not significant ($p = 0.33$; chi square).

Discussion

Mutations in the tumor suppressor gene p53 are present in over 90% of pelvic serous cancers, which are aggressive neoplasms that are typically discovered after spread to the peritoneum has occurred.²² The late stage of tumor presentation has fostered the impression that serous carcinogenesis evolves via rapid transitions from benign to malignant epithelium in the ovarian cortex or surface epithelium.¹ However, the past few years has witnessed a greater attention to the distal fallopian tube, where not only non-invasive (intraepithelial) serous carcinomas but a serous cancer precursor candidate, termed the p53 signature, has been described.^{6-12, 14} The p53 signature, like tubal intraepithelial carcinoma, involves secretory epithelial cells, contains p53 mutations, evidence of DNA damage, location in the distal fallopian tube, continuity with intraepithelial carcinoma, and association with decreased parity.^{5 14-17} With the increased awareness that the distal tube can give rise to both non-invasive (intraepithelial) carcinomas and a credible precursor to serous cancer (p53 signatures), irrespective of genetic risk, the field of ovarian cancer investigation has been expanded to include the distal fallopian tube as an important site of tumor origin.

Because p53 signatures have been associated with p53 mutations, and presumably arise via DNA damage, we addressed the hypothesis that in patients with germ-line mutations in p53, p53 signatures might be increased in frequency. This study shows, for the first time, that the distal fallopian tubes of women with LFS are exquisitely prone to developing p53 signatures identical to those described in women without LFS, including women with BRCA1/2 mutations and the general population.^{14 20} Relative to the endometrium and proximal fallopian tube, the frequency increases markedly in the region of the fimbria, where the number of p53 signatures exceeds by an order of magnitude the frequency observed in women without germline p53 mutations. Although fallopian tubes from only two cases of LFS were available for this study the high number of p53 signatures is in dramatically contrast to our prior findings in over 300 fallopian tubes from women with benign and malignant conditions, with and without increased (BRCA) genetic risk.¹⁵ In these latter groups, the number of p53 signatures encountered following extensive sectioning and immunostaining of an entire distal fallopian tube rarely exceeds three. The intense staining for p53 seen in the p53 signatures of LFS is in sharp contrast to the background epithelium of tubes from these women, suggesting that a constitutive point mutation in the p53 gene will not result in delayed degradation (and accumulation) of p53 protein.²³ Most of the foci sampled contained LOH of the second allele, albeit in lower frequency than observed in the serous carcinomas. This could be a function of the small DNA samples obtained by LCM and potential difficulties in generating gene product that is representative of the biologic process.

P53 mutations are integral to pelvic serous cancers and the role of germline or acquired BRCA1/2 mutations - followed by gene inactivation- on serous cancer development, is irrefutable. From 70-80% of ovarian tumors from BRCA1/2 mutation carriers exhibit LOH of the wild type allele.²⁴ A similar frequency of BRCA1/2 LOH is seen in sporadic serous carcinomas.²⁵ Prior functional inactivation of p53 appears critical to cell survival when LOH occurs in the wild-type BRCA allele^{26 27} and supports the models put forth by Knudson and Weinberg, in which two or more genetic events are required to generate the malignant phenotype.^{28 29} The existence of both p53 signatures and smaller numbers of p53 positive nuclei, including single strongly p53-positive cells, suggests that loss of p53 function in the context of the genotoxic injury is followed by variable turnover of the p53 positive cells. Whether this diversity in the number of p53 positive cells in a p53 signature reflects relative inhibition of cell expansion following the genotoxic insult, or epithelial cell targets with inherently different growth potentials, is unknown. The answer to this question might shed light on cell survival and “stemness” within the secretory cell population of the fallopian tube, similar to other models.³⁰

Figure 4 depicts a model for a serous carcinogenic sequence that takes into account sequential inactivation of both p53 and BRCA1/2. These events are triggered by DNA damage under mechanisms that remain to be elucidated and likely require the participation of additional oncogenic or tumor suppressor pathways (D. Dinulescu, personal communication). An intriguing hypothesis that is supported by both the LFS and BRCA1/2 model is that loss of p53 function in the context of a sub-morbid genotoxic insult is followed in many instances by continued - albeit gradual- cell turnover, leading to the p53 signature; a second event(s), inactivation of BRCA, would be necessary for the transition to a proliferative and potentially malignant lesion (intraepithelial carcinoma). A striking feature of this model is the apparent lack of a connection between the first (p53 inactivation) and second (BRCA1/2 inactivation) steps in this process. P53 signatures are common and highly abundant in tubes from the general population and women with LFS respectively, neither of which is particularly prone to ovarian cancer. This leaves the mechanism of BRCA1/2 inactivation a critical question, particularly in women who do not harbor inherited mutations in the gene. While this model will require refinement, it is becoming increasingly clear that

the initial events in the pathogenesis of pelvic serous cancer often occur in the distal fallopian tube, involve inactivation of the p53 locus and could predate malignancy by years. If this model is validated, it will establish the existence of an extended time span between a microscopically and genetically discernible precursor and the onset of serous malignancy. This interval could be relevant to not only our perceptions of the timing of serous cancer development, but to its site of origin and, ultimately, prevention.

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1. p53 signatures in a distal fallopian tube segment from two patients with Li-Fraumeni syndrome (LFS) (A&B) are characterized by strong linear nuclear p53 localization in secretory cells. The number of cells demonstrating nuclear DNA proliferation is low (C).

2.

A p53 signature at higher power demonstrates normal morphology (A) and strong p53 nuclear staining (B). Punctate nuclear staining with an antibody to H2AX is characteristic of localization of γ -H2AX (C, arrows). Another p53 signature (D) stains strongly for bcl-2, a secretory cell marker (E).

3. Comparison of deletion chromatograms from control epithelia (no LOH, upper) and a p53 signature (LOH, lower). The sequence chromatogram of the heterozygous LFS deletion 482_487delCCATCT is shown in panel A. The position of the deletion is marked by the arrow and both wild type and mutant alleles are clearly present (as demonstrated by the presence of sequence from both alleles superimposed after cDNA position 481). The sequence chromatogram from sample LFS10 is shown in panel B. Only the mutant allele is present demonstrating loss of heterozygosity of the wild type allele.

4.

A model for serous carcinogenesis in the distal fallopian tube, genotoxic stress imposed on the secretory cells (ovals) produces a p53 mutation followed by LOH of the non-mutated allele, with accumulation of p53 protein. Capacity of the affected cell for expansion is variable. Functional loss of BRCA1/2 via one or more mechanisms is a necessary component of progression to malignancy and is increased in women with inherited heterozygous BRCA1/2 mutations.

Table 1

p53 mutation and LOH analysis of Li-Fraumeni syndrome (LFS) associated p53 signatures and tumor tissues (T) from pelvic serous carcinoma (PSC)

Sample	Mutation	cDNA Δ	AA Δ	LOH
LFS1	482_487delCCATCT	482_487	NA	
LFS2	482_487delCCATCT	482_487	NA	
LFS3	482_487delCCATCT	482_487	NA	
LFS4	482_487delCCATCT	482_487	NA	LOH
LFS5	482_487delCCATCT	482_487	NA	
LFS6	482_487delCCATCT	482_487	NA	
LFS7	482_487delCCATCT	482_487	NA	LOH
LFS8	482_487delCCATCT	482_487	NA	LOH
LFS9	482_487delCCATCT	482_487	NA	LOH
LFS10	482_487delCCATCT	482_487	NA	LOH
LFS11	482_487delCCATCT	482_487	NA	LOH
PSC11T	c.488A>G,p.Y163YC	488A>G	Y163C	LOH
PSC12Ta	c.683_686delACTG	683del4	NA	LOH
PSC12Tb	c.683_686delACTG	683del4	NA	LOH
PSC13T	48DelCCA	48del3	R248W	LOH
PSC14T	c.742C>CT,p.R248RW	742C>T	NONE	
PSC15T	c.578A>G,p.H193R	578A>G	H193R	LOH
PSC16T	439delG	439delG	NA	LOH
PSC17T	c.949delC	949delC	NA	LOH
PSC18T	c.988delC	988delC	NA	LOH
PSC20T	c.747G>T,p.R249S	747G>T	R249S	
PSC21T	c.818G>GA,p.R273RH	818G>A	R273H	
PSC22T	c.659A>G,p.Y220C	659A>G	Y220C	LOH
PSC23T	c.406C>CG,p.Q136QE	406C>G	Q136E	
PSC24T	c.772_79elGAAGACTCCAGGTCAGGAGCC	772del20	NA	LOH
PSC25T	c.524G>GA,p.R175RH	524G>A	R175H	
PSC26T	c.844C>G,p.R282G	844C>G	R282G	LOH
PSC27T	IVS560-3T>TG/c.596G>GA,p.G199GE	IVS560-3T>G	NA	
PSC28T	c.637C>CT,p.R213RX	637C>T	R213X	
PSC29T	c.711G>A,p.M237I	711G>A	M237I	LOH
PSC30T	c.838A>G	838A>G	R280G	LOH
PSC31T	c.438G>A	438G>A	W146X	LOH
PSC32T	c.734G>C	734G>C	G245A	LOH

Δ, change; LOH, mutation and loss of heterozygosity of the wild-type allele.