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Frequent mutations in the *RPL22* gene and its clinical and functional implications ☆

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HIGHLIGHTS

- Heterozygous mutation of the RPL22 mutation was very common in endometrioid endometrial cancers with defective DNA mismatch repair.
- It was not associated with stage, grade, race, lymphovascular space invasion and progression-free survival.
- RPL22 may be a driver mutation in development of endometrial cancer and assessment of the RPL22 knockdown may be warranted.

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ABSTRACT

Objective. To determine the frequency and spectrum of mutations in RPL22 a gene identified by The Cancer Genome Atlas (TCGA) as mutated in endometrioid endometrial cancer, and determine the relationship between RPL22 defects and clinicopathologic features.

Methods. Direct sequencing of the entire coding region of the RPL22 cDNA and exons 2/4 was performed in tumors with/without microsatellite instability (MSI). RPL22 expression was assessed by immunofluorescence microscopy in the KLE, RL952 and AN3CA cell lines, wildtype, heterozygous and homozygous mutants, respectively. Relationships between RPL22 mutation and clinicopathological features were assessed using Chi-squared analysis and Student's *t* test. Progression-free survival (PFS) was calculated from the date of diagnosis to the date of recurrence.

Results. A single nucleotide deletion in an A8 coding repeat was identified in exon 2 of the RPL22 gene in 116/226 (52%) of MSI-high tumors. No mutations were identified in MSI-stable tumors. Only 2% of the tumors expressed a homozygous A deletion. RPL22 mutation was not associated with stage, grade, race and lymphovascular space invasion. Women whose tumors harbored RPL22 mutations were significantly older (67 vs. 63 years, $p = 0.005$). There was no difference in PFS between patients with the wildtype and mutant genotypes.

Conclusions. RPL22 is frequently mutated in MSI-high endometrioid endometrial cancers. The A8 mutation identified was not reported in the whole exome sequences analyzed by the TCGA. The demonstration of frequent mutation in RPL22 may point to a limitation of the exome capture and next generation sequencing analysis methods for some mononucleotide string mutations. Functional assessment of the RPL22 knockdown may be warranted.

Introduction

Endometrial cancer is the most common gynecologic malignancy in the United States. An estimated 47,130 women will be diagnosed with uterine cancer in 2012 [1]. Although the majority of women will be diagnosed with localized disease with a 95% 5-year survival rate [2], a subset of patients have more aggressive tumors and present with advanced disease or develop recurrences after initial treatment. Prognosis for these women is significantly worse with 5-year overall survival of 40–60% for locally advanced disease and 20% for metastatic disease [3]. This subset of patients accounts for most of the estimated 8,010 endometrial cancer deaths in 2012 [1]. Few advances in the treatment of persistent and/or recurrent endometrial cancer have been made over the past decade. Elucidation of the genetic factors that underlie aggressive tumor behavior holds promise for the development of therapies that target this subset of endometrial cancers.

A major goal of the Cancer Genome Atlas (TCGA) initiative is to "further scientific understanding of cancer and create a comprehensive 'atlas' of the genomic changes involved in cancer" [4]. It is hoped that

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broader understanding of the genomic landscape of uterine cancers will provide opportunities to better diagnose, treat and prevent endometrial cancer. The ongoing Endometrial Cancer TCGA project has revealed hundreds of genomic abnormalities in endometrioid endometrial cancer tumors. Developing in vitro or in vivo models to assess the functional consequence of all candidate mutations/gene defects is prohibitively expensive. A first step to credentialing candidate genes is evaluation of mutations in representative endometrial cancer populations to assess the relationship between mutation and clinicopathologic features including outcome.

The TCGA identified the *RPL22* as a gene mutated in endometrioid endometrial cancer. Ribosomal Protein L22 (RPL22) is a component of the 60s subunit of the ribosome. Its function has not been extensively characterized. *RPL22* knockdown in a mouse model resulted in decreased lymphocyte counts due to impaired generation of αβ-lineage cells [5] and silencing of RPL22 expression using siRNA leads to inhibition of human pulmonary arterial smooth muscle cells [6]. A potential role for RPL22 in tumor development was suggested by a recent study that identified another member of the ribosomal protein family, RPL11, as a modulator of p53 pathway, a known contributor to tumorigenesis [7].

The TCGA identified several missense mutations in *RPL22* (personal communication). *RPL22* has two coding mononucleotide repeats (A_8 and A_6) that are potential sites for strand slippage mutations in tumors lacking DNA mismatch repair. The loss of DNA mismatch repair is a frequent event in endometrioid endometrial cancers, resulting in the accumulation of strand slippage mutations and tumor microsatellite instability (MSI). Defective DNA mismatch repair in colorectal cancer is associated with improved outcomes in a subset of patients [8–10]. Although ~30% of endometrioid endometrial cancers have MSI, the association between MSI and clinical outcomes is unclear. Multiple studies have attempted to determine the impact of MSI in the outcome of patients with endometrial cancer with conflicting results.[11–18]

In this study we assessed *RPL22* mutation and determined the relationship between mutation status and clinicopathologic features.

Materials and methods

Study participants and clinical data

Tumors and associated clinical and pathologic data were collected within the Division of Gynecologic Oncology at Washington University School of Medicine from 1991 to 2010 as previously described [18,19]. All enrolled participants consented to molecular analyses and follow-up monitoring as part of ongoing Washington University's Human Research Protection Office–approved research protocols (protocols 91–507 and 93–0828).

All cases underwent primary surgery by a gynecologic oncologist at Washington University School of Medicine/Barnes–Jewish Hospital. Surgical staging and tumor grade was assigned on the basis of International Federation of Gynecology and Obstetrics (FIGO) 2009 criteria. Specific treatment was individualized and left at the discretion of the patient's attending physician. Recommended follow-up was at 3-month intervals for the first 2 years and then 6-month intervals for at least 3 years. All suspected recurrences were histologically confirmed. Only endometrioid endometrial cancers were included in this study.

Tissue processing, MSI typing and preparation of nucleic acids

Tissue and blood specimens were obtained at the time of surgery, snap-frozen, and stored at −70 °C. DNA was prepared from tumors using proteinase K and phenol extraction or with the DNeasy Tissue Kit (Qiagen Inc, Valencia, CA). Normal DNA was extracted either from peripheral-blood leukocytes or uninvolved myometrium as previously described [18,20,21].

Genomic DNA from tumor tissues, normal endometrium and cell lines was extracted using the DNeasy Tissue kit (Qiagen, #69506). Total cellular RNA was prepared from tumors and cell lines using the Trizol reagent (Invitrogen, #10296-010). Complementary DNA (cDNA) was generated using 1 μg total RNA and the QuantiTect Reverse Transcription Kit (Qiagen, #205311).

Microsatellite analysis was performed using five National Cancer Institute consensus microsatellite markers (BAT25, BAT26, D2S123, D5S346, and D17S250). Standard definitions for MSI positivity were applied as previously described [22].

RPL22 mutation analysis

PCR amplification (cDNA or genomic DNA) was performed using either Amplitaq DNA Polymerase (Applied Biosystems, Carlsbad, CA) or Phusion High-Fidelity DNA Polymerase (New England Biolab, Ipswich, MA). PCR primers and conditions are demonstrated in Table 1.

Amplification products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced using ABI Prism BigDye 3.1 (Applied Biosystems, Foster City, CA). Sequence traces were evaluated using Sequencher v4.10.1 (Gene Codes Corporation, Ann Arbor, MI) and by visual inspection of individual chromatograms.

Cell culture

The AN3CA, HEC1A, KLE and RL952 endometrial cancer cell lines were purchased from the American Type Culture Collection. The Ishikawa and MFE296 cell lines were gifts from Dr. Stuart Adler (Washington University School of Medicine, Department of Internal Medicine) and Dr. Pamela Pollock (Queensland University of Technology, Brisbane), respectively. Cell lines were grown as previously described [22] and authenticated as described by Dewdney et al. [23].

Immunofluorescence

Cells were seeded on coverslips at a density of 5×10^4 and allowed to grow to 50–60% confluence. Cells were fixed with 4% formaldehyde and then permeabilized and blocked with a solution containing 0.5% Triton X-100, 2% BSA and 5% normal goat sera in PBS. Cells were incubated with a rabbit polyclonal against amino acids 17–122 of human RPL22 1:50 (sc-98857: Santa Cruz Biotechnology, Santa Cruz, CA) and subsequently with an Alex Fluor 488 secondary antibody 1:200 (A-11034: Life Technologies, Grand Island, NY). Nuclei were stained with TO-PRO-3 iodide (T3605: Invitrogen, Eugene, OR). Confocal imaging was done with a Nikon E800 Eclipse C1 microscope with a 20x objective, 488 and 633 nm lasers.

Table 1
PCR primers and conditions.

Product	Amplicon size	Forward primer	Reverse primer	Annealing temperature
Exon 2	398 bp	5′-GCCTCTTGGCCCTCTGGCAA-3′	5′-TGCCCACATTCCCAGGGTGAGA-3′	63 °C
Exon 4	575 bp	5′-CTCAGAGCCTTGGCTGGGTGTA-3′	5′-CCCGCATCTGCCTCCCCAATG-3′	63 °C
Full codon	706 bp	5′-GGCTCTTTGCGTCTGCGTAGTT-3′	5′-CCCGCATCTGCCTCCCCAATG-3′	63 °C

Statistical analysis

The relationship between *RPL22* mutation status and covariates was assessed using Chi-squared analysis, Fisher's exact test and Student's *t* test. Heterozygous and homozygous mutations were combined for analysis. Progression-free survival (PFS) was calculated from the time of diagnosis to the date of recurrence. Survivors without recurrence were censored at the date of last contact. A Cox regression model was designed to evaluate the impact of *RPL22* mutation on PFS controlling for stage and grade. The Kaplan–Meier product-limit method was used to estimate PFS. A p-value <0.05 was considered statistically significant for all analyses. All statistical analyses were performed using Stata v9.2, (StataCorp LP, College Station, TX).

Results

RPL22 mononucleotide repeat mutations

Direct sequencing of *RPL22* exons 2 and 4 in 23 MSI-high and 23 MSI-stable endometrioid endometrial tumors revealed mutation in the exon2 A_8 repeat (Ch1:6,257,792ΔA) in 12/23 (52%) of MSI-high cases. This mutation, 43delA, is not reported in the TCGA endometrial cancer mutation list whereas the missense mutations identified by the TCGA were not seen in this cohort.

All mutant cases were heterozygous for the 43delA mutation and none of the MSI-stable tumors carried the 43delA mutation. Sequencing matched normal DNAs from the mutated samples confirmed the delA mutation was somatic.

Sequencing cDNA encompassing the entire *RPL22* coding region from eight primary tumors carrying the 43delA mutation (all MSI-high) and nine MSI-stable tumors identified no additional mutations. Analysis of six endometrial cancer cells lines revealed wildtype sequence in the KLE cell line, heterozygous deletion of A in the A_8 repeat track in the HEC1A, Ishikawa and MFE cell lines, heterozygous deletion of two adenine residues in the A_8 repeat track in the RL952 cell line and homozygous deletion of a single A in the AN3CA cell line.

Direct sequencing of 203 additional MSI-high endometrioid endometrial cancers confirmed that the exon 2 A_8 mononucleotide repeat track is frequently mutated in endometrial cancers with defective DNA mismatch repair. Overall 116/226 (51.6%) tumors were heterozygous for the 43delA mutation. Four tumor specimens (1.8%) were homozygous for the A deletion mutation. Representative sequences of wildtype, heterozygous and homozygous deletions are presented in Fig. 1A.

The 43delA mutation is associated with loss of immunodetectable RPL22

To assess the effect of the *RPL22* mutation on protein expression we used immunofluorescence microscopy in endometrial cancer cell lines. The 43delA mutation involves one of four lysines at amino acids 13–16. Deletion of either one or two A nucleotides results in frameshift mutations at the 14th amino acid of the RPL22 protein leading to truncated proteins of 18 and 22 amino acid lengths, respectively.

Immunofluorescence using a polyclonal antibody to the RPL22 protein demonstrated the presence of the protein in the KLE and RL952 cell lines, wildtype and heterozygous mutants, respectively. AN3CA, a cell line homozygous for the 43delA mutation, had no immunodetectable *RPL22* (Fig. 1B). Western blot analysis confirmed the presence of RPL22 in endometrial cancer cell lines expressing the wildtype or heterozygous A deletion and absence in the AN3CA cell line (data not shown).

Clinicopathologic correlates

Follow-up data for 218/226 (96.5%) of the tumor samples were available and included in the clinical analysis. Two patients had perioperative mortalities unrelated to their disease while six patients did not follow-up after surgery and were excluded from the analysis.

RPL22 mutation was not associated with tumor stage, grade, patient race and lymphovascular space invasion, all of which are known to be associated with worse clinical outcomes. Mutation was, however, significantly associated with older age at diagnosis (67 vs. 63 years, $p=0.005$) (Table 2). Cox regression model evaluating the impact of the *RPL22* mutation on progression-free survival demonstrated no association between mutation status and outcome after controlling advanced stage and higher grade. (Hazard Ratio 0.95, 95% C.I.: 0.50–1.78). As expected, stage III/IV disease and grade 2/3 tumors were associated with hazard ratios of 3.11 (95% C.I.: 1.64–5.90) and 3.43

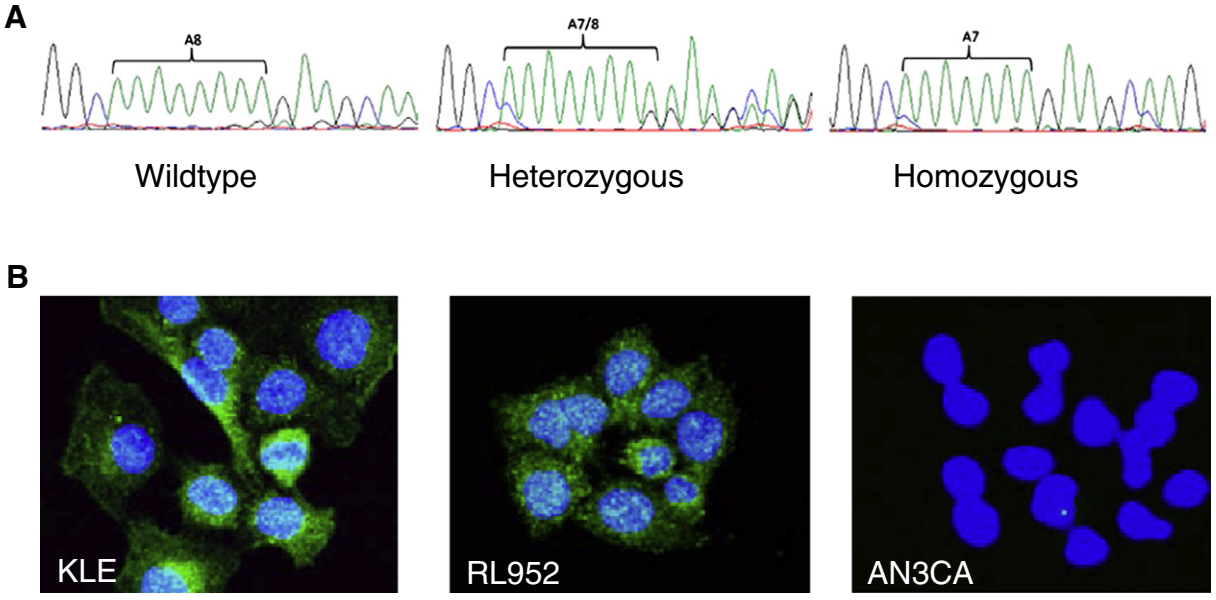

Fig. 1. RPL22 mutation analysis and expression. (A) Chromatograms demonstrating the wildtype *RPL22* sequence, heterozygous and homozygous deletions of a single nucleotide. (B) Immunofluorescence demonstrating RPL22 expression in the KLE (wildtype) and RL952 (heterozygous deletion) cell lines and absence of expression in the AN3CA (homozygous deletion) cell line.

Table 2
Clinicopathologic features of patients with wildtype and mutant *RPL22* genotypes.

Characteristic		RPL22 wildtype ($n=101$) n (%)	RPL22 mutant ($n=117$) n (%)	p
Age (years)				
	Mean (SD)	63 ± 11	67 ± 11	0.005
Race				
	White	93 (92)	102 (87)	
	All others	8 (8)	15 (13)	0.24
Stage				
	I	79 (78)	91 (78)	
	II	1 (1)	3 (3)	
	III	13 (13)	19 (16)	
	IV	8 (8)	4 (3)	0.37
FIGO grade				
	1	39 (3)	46 (39)	
	2	48 (48)	56 (48)	
	3	14 (14)	15 (13)	0.97
Lymphovascular space invasion				
	Absent	53 (50)	68 (57)	
	Present	53 (50)	52 (43)	0.32

(95% C.I.: 1.42–8.27), respectively, for recurrent or progressive disease. Kaplan–Meier curves for progression-free survival, by *RPL22* mutation status are presented in Fig. 2. No significant difference was seen between patients with the wildtype and mutant genotypes ($p=0.91$).

Discussion

The Cancer Genome Atlas has provided researchers with an unprecedented data resource that comprises the genomic landscape of all the tumor types studied to date. Translating Cancer Genome Atlas into clinically relevant interventions presents a major challenge. Extensive data curation and follow-up will be required to distinguish signal from noise in the complex genomes of endometrial cancers.

In this study we identified *RPL22* as a gene frequently mutated in MSI-high endometrioid endometrial cancers. The single base deletion identified in 52% of MSI-high tumors (estimated 15% of endometrioid cancer overall) in this study was not reported in the whole exome sequences analyzed by the TCGA and we were not able to demonstrate any of the mutations identified by the TCGA. The fact that the 43delA was not reported in the endometrial cancer TCGA data set may point to the relative insensitivity of exome capture/next generation sequencing and analysis methods for alterations in mononucleotide tracks.

Approximately 50% of endometrial cancers with microsatellite instability in our cohort are heterozygous for a mutation resulting in a truncated protein. Only 2% of the tumors were homozygous mutants. The *RPL22* mutation we identified results in a loss of function and we demonstrated the absence of the RPL22 protein in a homozygous deleted endometrioid cell line. The role the *RPL22* mutation plays in tumorigenesis may be small given the lack of significant association between mutation status and relevant clinicopathologic variables including outcome. Because of the low prevalence of the homozygous mutation, our study is limited in its ability to assess clinical correlates of the homozygous mutant and we combined heterozygous and homozygous mutations.

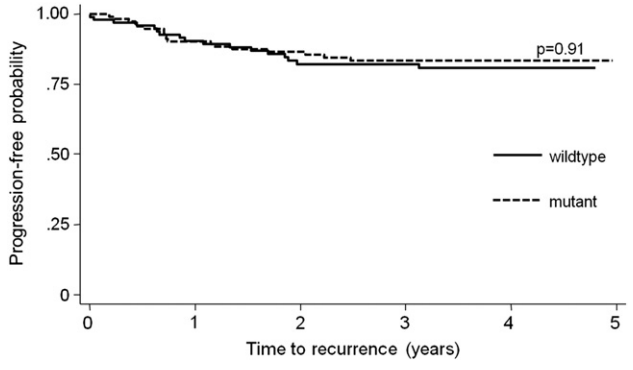

Fig. 2. Kaplan–Meier survival curves demonstrating no difference in progression-free survival between patients with wildtype and mutant *RPL22*.

Our findings suggest two possibilities in the understanding of the *RPL22* mutation. *RPL22* may be a "passenger mutation" with no selective benefit in the established tumor. However, the high prevalence of this mutation suggests that the *RPL22* mutation may play a more integral role in the development of endometrial cancer. Endometrioid endometrial cancer has histologically recognized precursors with progression from simple hyperplasia to complex hyperplasia to complex atypical hyperplasia finally resulting in the development of adenocarcinoma. Multiple mutations have been identified with a prevalence ranging 10–50% including PTEN, TP53, loss of DNA mismatch repair and KRAS [24–26]. The association between these mutations and outcomes has been conflicting. Similar to these genes, *RPL22* as a ribosomal protein may be a selected mutation in the progression to endometrioid endometrial cancer. Thus, functional assessment of the knockdown of the *RPL22* protein may be warranted.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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