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Mutational Analysis of *ZFY* in Sporadic Parathyroid Adenomas

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Context: The molecular pathogenesis of sporadic parathyroid adenomas is incompletely understood, with alterations in *cyclin D1/PRAD1* and *MEN1* most firmly established as genetic drivers. The gene encoding the X-linked zinc finger protein (*ZFX*) has recently been implicated in the pathogenesis of a subset of parathyroid adenomas after recurrent, hotspot-focused somatic mutations were identified. *ZFX* escapes X inactivation and is transcribed from both alleles in women, and a highly homologous gene encoding the Y-linked zinc finger protein (*ZFY*) provides dosage compensation in males.

Objective: We sought to investigate the role of *ZFY* mutation in sporadic parathyroid adenoma.

Intervention: Polymerase chain reaction and Sanger sequencing were used to examine DNA from typically presenting, sporadic (nonfamilial, nonsyndromic) parathyroid adenomas from male patients for mutations within the *ZFY* gene.

Results: No mutations were identified among 117 adenomas.

Conclusions: The absence of *ZFY* mutations in this series suggests that *ZFY* rarely, if ever, acts as a driver oncogene in sporadic parathyroid adenomas. The apparent differences in tumorigenic capabilities between the closely related zinc finger proteins ZFX and ZFY suggest that structure-function studies could represent an opportunity to gain insight into neoplastic processes in the parathyroid glands.

Freeform/Key Words: ZFY, ZFX, parathyroid, oncogene

Single parathyroid adenomas, the most common cause of primary hyperparathyroidism, are well-differentiated, benign, monoclonal tumors that give rise to hypercalcemia through excessive secretion of parathyroid hormone. Recurrent, clonally selected driver mutations in the cyclin D1 proto-oncogene and the *MEN1* tumor suppressor gene are established pathogenetic contributors in a subset of these tumors. Additional strong evidence (genetic plus functional) supports lesions of the *CDKN1B*/p27 cyclin-dependent kinase inhibitor (CDKI) gene, other CDKI genes, and *CDC73/HRPT2* as rare genetic contributors to sporadic, nonfamilial parathyroid adenoma formation [1–6], and evidence for the rare involvement of candidate parathyroid oncogenes *EZH2* and *CTNNB1* has also been reported [7–9]. However, our understanding of the molecular

Abbreviations: CDKI, cyclin-dependent kinase inhibitor; PCR, polymerase chain reaction; ZFX, X-linked zinc finger protein; ZFY, Y-linked zinc finger protein.

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pathogenesis of these tumors remains incomplete. Recently, compelling genetic evidence implicated the X-linked zinc finger protein (ZFX), a Kruppel C2H2-type zinc finger protein that has been reported to have a regulatory role in embryonic stem cell renewal, as a likely driver of parathyroid tumorigenesis. Exome sequencing and subsequent targeted validation sequencing revealed mutations in six of 130 sporadic parathyroid adenomas [10]. These mutations were strikingly specific and restricted in their locations, consistent with hypermorphic or neomorphic function; they affect one of the same two codons, 786 or 787, which encode two highly conserved arginine residues in the functionally critical 13th (C-terminal most) zinc finger domain. All of these substitutions were also predicted, by SIFT, to alter the functionality of the ZFX protein. Importantly, *ZFX*, situated on the X chromosome, escapes X inactivation and is transcribed from both alleles in females; in males the highly homologous zinc finger protein encoded by the Y chromosome (*ZFY*) is expressed to provide dosage compensation [11]. ZFX and ZFY are highly homologous, sharing 92% of their protein sequence identities overall, and 97% of their identities in the 13th zinc finger domains [11]; in addition, ZFY, like ZFX, is expressed in many tissues, including the parathyroid glands [12]. Because of the close relationship between these genes, we hypothesized that mutations in *ZFY* could potentially have a tumorigenic role in hyperparathyroidism similar to that of mutations in *ZFX*. We also noted that one *ZFY* mutation has been reported in a colon carcinoma (according to the Catalogue of Somatic Mutations in Cancer). Thus, we sought to investigate the role of *ZFY* in typically presenting sporadic parathyroid adenomas from male patients.

1. Materials and Methods

A. Patients and Samples

Tumor samples were obtained from 117 male patients who had undergone parathyroidectomy for primary hyperparathyroidism with typical presentations; these tumors were surgically and histopathologically proven as single parathyroid adenomas with no atypical and/or malignant features. No patient had a family and/or personal history that was suggestive of familial and/or syndromic hyperparathyroidism. All samples were obtained with informed consent in accordance with institutional review board–approved protocols. Genomic DNA was extracted from fresh, frozen tissue by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation.

B. Polymerase Chain Reaction and Sanger Sequencing

Primers were designed for *ZFY*'s seven coding exons (Table 1), such that intron regions at the splice junctions were also examined. The polymerase chain reactions (PCRs) were carried out in 20-μL reaction volumes, containing 25 ng of sample DNA, 12 μL of deionized H_2O, 2 μL of 10× PCR buffer (Applied Biosystems, Austin, TX), 200 μM of deoxynucleotide triphosphates, 1.2 μL of $MgCl_2$, 1 μM of forward and reverse primer, and 1.5 mM of Taq Gold (Applied Biosystems, Foster City, CA). The PCR reactions were performed by incubating at 95°C for 10 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds (60°C for exon 7), and 72°C for 1 minute, and a final elongation step of 72°C for 10 minutes. The PCR products were purified with ExoSAP-IT (Affymetrix, Santa Clara, CA) and were sequenced using standard Sanger sequencing methodology and the same primers used for PCR (GeneWiz, South Plainfield, NJ). Sequence data were analyzed using the Sequencher (Gene Codes, Ann Arbor, MI) DNA sequence analysis software to align sequences obtained from tumor DNA to the reference sequence.

2. Results

A total of 117 parathyroid adenomas were tested for sequence variants in the coding region of *ZFY*. One variant was found, which represented a single nucleotide polymorphism

Table 1. Primer Sets for *ZFY* Sanger Sequencing

Exon	Forward	Reverse	Expected Product Size (bp)
1	AGAGAAAGGCCGTCCTGCAGC	CTTCGCGGACATTTACATAGC	360
2	CCCATGTATCCCTGGAAATC	ACAGGACCTAAGTATGGACC	446
3 (a)	GAGGAACACCAAGGACATAG	TTCTGGCATAGACATTGAGG	400
3 (b)	CAAGTGCTGGACTCAGATGT	TGTTAGAAAACACAGGTTTC	295
4	AACAAAGATGACATATGTCC	CATTTGGTTGAAACATTTGG	342
5	ATACACTAAAACGTGTAAGC	TGATGGGTTGATAGGTGCAG	278
6	CTGTGTGATCTCTGTAAACC	AGAAACCACTTTCAACAGTG	297
7	TGTCATTCATGAGTGTCAGG	AATACATGTGGCCTACTAGC	441
8 (a)	ATTCATGAGGAGACCAGAAG	GAGGTGGCGATTCAATAACC	493
8 (b)	AAAAGGGGCCAACAAAATGC	TTCTGAAGGCCTGTGAAAGC	454
8 (c)	CACATCAGTGTTTGCATTGC	TCTGCTTCAAGGCCAACATC	431
8 (d)	ACCTTCAGAAAAGAACCAGC	GGCCTGTGAAAGCCTTTCTCG	362

Abbreviations: bp, base pair.

(rs111915341) in the intronic region preceding exon 5 (Table 1). If the true frequency of *ZFY* mutation in parathyroid adenoma was comparable to that of *ZFX* (6 of 130; 4.6%), this sample size of 117 was well powered to detect such mutations, with a statistical power above 80%.

3. Discussion

Recurrent somatic hotspot mutations identified in *ZFX* have provided compelling genetic evidence implicating the X-linked zinc finger protein in the molecular pathogenesis of parathyroid adenoma [10]. Importantly, *ZFX* escapes X inactivation and is transcribed from both alleles in women, whereas a highly homologous gene encoding the Y zinc finger protein (*ZFY*) provides dosage compensation in males [11]. As such, it is quite plausible that *ZFY* could harbor driver mutations that contribute to the molecular pathogenesis of parathyroid adenomas, similar to mutations found in *ZFX*.

However, this study did not identify *ZFY* somatic mutations or sequence variants of likely pathogenicity in any of the 117 parathyroid adenomas that were analyzed. Our sample size was well powered to detect a *ZFY* mutation if the frequency of such events was similar to that of *ZFX*, and we therefore conclude that the mutational frequency of *ZFY*, if mutations occur at all, is likely much lower than that of *ZFX*. Our observations imply that mutations in *ZFY* (even those corresponding to the ZFX hotspot) fail to yield a selective advantage to parathyroid cells that may randomly acquire a mutation, and that the few surrounding differences in the sequences of the ZFX and ZFY proteins may be important in determining their relative oncogenicity. Thus, structure-function studies of these proteins and mutations could yield insights into neoplastic mechanisms in the parathyroid cell context.

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