RESEARCH LETTER

Two distinct classes of thymic tumors in patients with MEN1 show LOH at the MEN1 locus

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Abstract

Patients with the multiple endocrine neoplasia type 1 (MEN1) syndrome carry germline heterozygous loss-of-function mutations in the MEN1 gene which predisposes them to develop various endocrine and non-endocrine tumors. Over 90% of the tumors show loss of heterozygosity (LOH) at chromosome 11q13, the MEN1 locus, due to somatic loss of the wild-type MEN1 allele. Thymic neuroendocrine tumors (NETs) or thymic carcinoids are uncommon in MEN1 patients but are a major cause of mortality. LOH at the MEN1 locus has not been demonstrated in thymic tumors. The goal of this study was to investigate the molecular aspects of MEN1-associated thymic tumors including LOH at the MEN1 locus and RNA-sequencing (RNA-Seq) to identify genes associated with tumor development and potential targeted therapy. A retrospective chart review of 294 patients with MEN1 germline mutations identified 14 patients (4.8%) with thymic tumors (12 thymic NETs and 2 thymomas). LOH at the MEN1 locus was identified in 10 tumors including the 2 thymomas, demonstrating that somatic LOH at the MEN1 locus is also the mechanism for thymic tumor development. Unsupervised principal component analysis and hierarchical clustering of RNA-Seq data showed that thymic NETs formed a homogenous transcriptomic group separate from thymoma and normal thymus. KSR2 (kinase suppressor of Ras 2), that promotes Ras-mediated signaling, was abundantly expressed in thymic NETs, a potential therapeutic target. The molecular insights gained from our study about thymic tumors combined with similar data from other MEN1-associated tumors may lead to better surveillance and treatment of these rare tumors.
WT allele resulting in loss of heterozygosity (LOH) at chromosome 11q13, the \textit{MEN1} gene locus (Thakker et al. 2012). An uncommon manifestation of \textit{MEN1} is thymic neuroendocrine tumor (thymic NET), also referred to as thymic carcinoid, which in \textit{MEN1} patients is a major cause of mortality (Goudet et al. 2010). In contrast to the other frequent NETs in \textit{MEN1} patients (pancreas, parathyroid, and pituitary), LOH at the \textit{MEN1} locus has not been demonstrated in \textit{MEN1}-associated thymic tumors (Teh et al. 1998, Gibril et al. 2003, Pan et al. 2005). Therefore, it is generally thought that thymic tumor development in \textit{MEN1} patients is dependent on other somatic molecular events rather than a second hit to the \textit{MEN1} gene, and its pathogenesis is largely unknown. The lack of knowledge of its pathogenesis limits the ability to explore therapies directed at this tumor, which is the most aggressive of all \textit{MEN1}-associated tumors. The goal of this study was to investigate the molecular events contributing to thymic tumor development in our well-characterized cohort of \textit{MEN1} patients by evaluating LOH at the \textit{MEN1} locus and by utilizing transcriptomics to identify possible molecular hits that may lead to tumor development and could be considered for targeted therapy.

This study was conducted under the approval of the Institutional Review Board of the National Institutes of Health (NIH). All subjects who participated in this study provided written informed consent. We performed a retrospective chart review of 294 patients with \textit{MEN1} germline mutations from 2 long-standing natural history protocols at our institute (NCT00001277 and NCT00001345) and identified 14 patients (4.8%) with thymic tumors (12 males and 2 females), including 6 patients previously reported (Gibril et al. 2003).

Histology and immunoreactivity for neuroendocrine differentiation markers (chromogranin A and synaptophysin) confirmed the neuroendocrine phenotype in 12 tumors. Two tumors were confirmed as thymoma, of which one showed neuroendocrine differentiation. Formalin-fixed paraffin-embedded (FFPE) tissue sections were obtained from thymic tumors of patients who underwent surgery at the NIH Clinical Center between 1980 and 2020. FFPE sections of normal thymus tissue were obtained from commercial sources (Zyagen, San Diego, CA, USA; US Biomax, Derwood, MD, USA) \((n=2)\), from NIH patients who underwent parathyroid surgery and removal of the normal thymus \((n=3)\), or adjacent normal of a patient with \textit{MEN1} who underwent surgery for thymic carcinoid \((n=1)\). Microdissection of the FFPE tissue sections was performed to separate the tumor tissue from the stroma. DNA and RNA were isolated using DNAstom FFPE DNA and RNAstom FFPE RNA extraction kits (Cell Data Sciences, Fremont, CA, USA), respectively. Genomic DNA was isolated from whole blood samples using the iPrep Purification Instrument (Thermo Scientific). LOH at the \textit{MEN1} locus in tumor DNA was ascertained by Sanger sequencing of PCR products encompassing the region with germline \textit{MEN1} mutation or PCR-based analysis of blood and tumor DNA with polymorphic microsatellite markers at chromosome 11q13, using previously published or newly designed primers. All primer sequences are available upon request. RNA-Sequencing (RNA-Seq) and analysis was performed by a commercially available sequencing service (QuickBiology, Pasadena, CA, USA). The trimmed mean of M values (TMM) method in edgeR package was used to normalize the gene expression, and differentially expressed genes were identified. Genes showing altered expression with edgeR multiple testing adjusted \(P\) value \(< 0.05\) and more than 1.5-fold changes were considered differentially expressed.

The 13 different heterozygous \textit{MEN1} germline mutations identified in the 14 cases with thymic tumors (patients 4 and 8 were from the same family) were scattered throughout the entire \textit{MEN1} coding region without specific clustering (Fig. 1A and B). However, we observed a preponderance (85%) of protein-truncating mutations (frameshift and nonsense). Five of the 13 mutations were not reported previously (patients 4, 6, 9, 12, and 13).

Somatic LOH at the \textit{MEN1} locus was identified in 10 tumors including the 2 thymomas (7 through direct Sanger sequencing and an additional 3 using polymorphic markers) (Fig. 1C, D, and E). Though thymic NETs have long been associated with \textit{MEN1}, this is the first time that LOH at the \textit{MEN1} locus has been shown in multiple thymic tumors demonstrating that, despite previous unsuccessful attempts to find LOH (Teh et al. 1998, Gibril et al. 2003, Pan et al. 2005), the Knudson two-hit hypothesis also applies to the thymic tumors of \textit{MEN1} syndrome, with LOH as the apparent mechanism of tumorigenesis (Knudson 1971). In addition, we showed that not only thymic NETs but also thymomas should be considered as a manifestation of the \textit{MEN1} syndrome. It is unclear why \textit{MEN1} LOH was not detected in these tumors in previous studies. One possibility is the fact that these tumors are interspersed with normal vascular and stroma tissue due to their invasiveness. Other possible reasons that may have hindered the definitive assessment of molecular abnormalities in previous studies could be the sampling of tissue sections that lack the representative tumor part or the lack of microdissection of the tumor tissues.
Whole transcriptome analysis of MEN1-associated thymic tumors has not been reported. We performed RNA-Seq analysis of 11 thymic NETs, 2 thymomas, and 6 normal thymuses. Unsupervised principal component analysis and hierarchical clustering based on all genes expression showed that the 19 samples separated into at least 2 clusters. Thymic NETs formed a homogenous group distinct from the two thymomas which showed clustering similar to but likely distinct from the normal thymus samples (Fig. 2A and B).

Of 19,657 identified transcripts from protein-coding genes, a set of 8876 genes was significantly differentially expressed between the thymic NETs and the normal thymus groups. Of these, 4692 genes were upregulated and 4184 were downregulated in thymic NETs compared to normal thymuses (Fig. 2C and D). Relative to the 6 normal thymuses and 2 thymomas, the 11 thymic NETs had significantly higher mRNA for neuroendocrine markers (more than 100-fold, \( P < 0.02 \)) chromogranin A, chromogranin B, neuron-specific enolase, and synaptophysin, confirming the neuroendocrine origin (Fig. 2E). Curiously, the thymoma with neuroendocrine differentiation had high chromogranin B but without significant expression of other neuroendocrine markers. Among the tumors with or without LOH at the MEN1 locus, there was no significant difference in MEN1 gene expression. Further analysis may help to determine whether the mutant transcript is overrepresented to compensate for the lack of normal MEN1 transcript. Immunostaining for menin was not performed.

NETs are known to express somatostatin receptors (SSTRs) that allow tumors to be imaged as well as treated with somatostatin analogs due to their antiproliferative effects. Given the clinical relevance of SSTRs, we evaluated the expression of the five known SSTR-encoding genes in the RNA-Seq data of our patient samples. SSTR1, SSTR2, and SSTR3 were most frequently expressed across the thymic NETs cohort, while SSTR4 was not detectable in any of the 11 tumors by Sanger sequencing (\( Y = \) yes, \( N = \) no) or the indicated polymorphic microsatellite markers at the MEN1 locus (\( R = \) retained, \( N = \) non-informative, ND = not done). LOH in the tumors of patients 7, 10, and 11 was not detected by Sanger sequencing of tumor DNA and could not be assessed with polymorphic microsatellite markers because blood DNA was not available. Previously, LOH was evaluated by polymorphic microsatellite markers in four of six tumors included in this study (patients 1, 7, 10, and 11) (Gibril et al. 2003). Regarding lack of LOH in the tumor of patient 1 in the previous study, it is highly likely that a different FFPE tissue block of the surgically removed tissue was used in this study.
positive in 91% \( (n = 11/12) \) and 100% \( (n = 3) \), respectively, consistent with SSTR expression.

Out of the 4692 significantly upregulated differentially expressed genes in thymic NETs, KSR2 (Kinase Suppressor of Ras 2) was the most significantly upregulated (390-fold compared to normal thymuses, \( P < 0.01 \)) (Fig. 2D and G). The KSR2 gene encodes for an intracellular protein that acts as a molecular scaffold for the Ras/Raf/MEK/ERK signaling pathway to promote Ras-mediated signaling. KSR2 connects Raf to its substrates MEK and ERK, and it is also involved in AMP kinase, RET, and Notch signaling (Fernandez et al. 2012). KSR2 has been reported to play a role in many diseases including early onset obesity, metabolic syndrome, insulin resistance, non-small cell lung cancer, melanoma, endometrial cancer, and invasive ductal carcinoma of the breast. Potential targeted therapy with available small molecule inhibitors of KSR2 has been an area of significant interest (Dhawan et al. 2016).

In conclusion, we show for the first time that, somatic LOH at the MEN1 locus is the mechanism for thymic tumor development in MEN1 patients. LOH was also found in the thymomas suggesting that they are also a manifestation of the MEN1 syndrome. One limitation of our study is that not all tumor samples showed evidence of LOH at the MEN1 locus, likely because of incomplete separation of tumor and non-tumoral cells. We found that thymic NETs formed a homogenous transcriptomic group that showed separation from thymomas and normal thymus samples. Separate clustering of tumors may indicate that thymic NETs and thymomas result from alterations in distinct
pathways downstream of loss of the MEN1-encoded protein menin. Another limitation of our study is that we compared the transcriptome of thymic tumors to normal thymuses, given that no cell-of-origin has been shown for MEN1-associated thymic tumors. Further studies comparing our valuable dataset of thymic tumor RNA-Seq with the cell-of-origin will be informative to reveal gene signatures specific to thymic tumors and the precise molecular mechanisms of thymic tumor pathogenesis. The molecular insights from our study coupled with an understanding of the modifying factors that result in the unique and aggressive behavior of thymic NETs compared to the behavior of the other NETs in MEN1 patients may lead to better surveillance and treatment for patients with thymic tumors, which is very limited at present.

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