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ORIGINAL ARTICLE

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Heightened *BRAF* and *BRAF* pseudogene expression levels in 2 Japanese patients with Erdheim-Chester disease

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Abstract

Background: Erdheim-Chester disease (ECD) is a rare form of non-Langerhans cell histiocytosis with multi-organ involvement. Many cases have mutations in the *BRAF* and other genes involved in the MAPK activation pathway. Pseudogenes, which regulate their parental genes post-transcriptionally, are overexpressed in various tumors, but we found no previous reports of high pseudogene expression in ECD.

Methods: We evaluated the xanthoma tissues of two patients with ECD. *BRAF* from the genomic DNA of the tissue was amplified by the polymerase chain reaction (PCR). The amplified fragment was directly sequenced to search the *BRAFV600E* mutation. Real-time PCR was performed to amplify cDNA via primer sets specific for either *BRAF* or BRAF pseudogenes.

Results: The first case, with advanced stable ECD, expressed high levels of *BRAF* pseudogenes and *BRAF*, and a low frequency of the *BRAFV600E* mutation. The second case, with early active ECD, showed high expression levels of both *BRAF* pseudogenes and *BRAF* gene but no *BRAFV600E* mutation.

Discussion: In the early-stage of ECD, high levels of BRAF pseudogene expression may boost BRAF expression, promoting the proliferation of xanthomas. The BRAF-V600E mutation may be associated with advanced stable stage.

Conclusion: This is the first report of ECD without a *BRAFV600E* mutation and with elevated *BRAF* gene and *BRAF* pseudogene expression. The etiologic ramifications of BRAF or BRAF pseudogenes in patents with ECD will be the focus of future study.

KEYWORDS

BRAF gene, *BRAF* pseudogene, Erdheim-Chester disease, xanthoma

1 | INTRODUCTION

Erdheim-Chester disease (ECD) is a rare form of non-Langerhans cell histiocytosis with multiple organ involvement. ECD was first reported as a lipoid granulomatosis by Jakob Erdheim and William Chester in 1930,[1] but it was later demonstrated to be non-Langerhans cell histiocytosis.

Consensus guidelines for the diagnosis of ECD were published in 2014.[2] The radiographic finding of symmetric diaphyseal and metaphyseal osteosclerosis in the legs is present in 95% of patients with

ECD, but only 50% of patients describe bone pain. Other hallmark manifestations are periaortic sheathing ("coated aorta"); pericarditis; myocardial infiltration; pulmonary interlobular septal thickening; perinephric infiltrates ("hairy kidneys"); central nervous system involvement; orbital infiltration, resulting in exophthalmos; endocrinopathies, such as diabetes insipidus; and skin xanthomas and xanthelasmas. It is difficult to diagnose ECD without cutaneous findings in early-stage disease because symptoms are often nonspecific and subjective. Sometimes ECD is only diagnosed at autopsy.[3–6]

Dermatologic findings of ECD include xanthomas of the face, neck, axilla, and trunk, which are found in approximately one-third of people with ECD.[7] Xanthelasmas appear in approximately 25% of patients with ECD.[8] The presence of skin manifestations leads to earlier diagnosis because the lesions are easily identified by dermatologists, and it is easier to obtain a biopsy from the skin than from other organs.

Histopathologic findings of ECD demonstrate infiltration of typically foamy or lipid-laden histiocytes with admixed or surrounding fibrosis. Touton giant cells are often present. The histiocytes in ECD are tissue macrophages devoid of S-100, CD1a, and CD207 surface markers but showing CD68, CD163, and Factor XIIIa immunoreactivity. They also express a pattern of inflammatory cytokines and chemokines, including interferon alpha (IFN-α),[9] interleukin-12 (IL-12),[9] monocyte chemotactic protein-1 (MCP-1),[9,10] interleukin-1 (IL-1),[9] and tumor necrosis factor-alpha (TNF-α).[11] We previously reported that xanthomas proliferated in the region of dermatitis in a patient with ECD.[12] Indeed, ECD was previously considered an inflammatory disease, until it was classified as a hematopoietic neoplasm of histiocytic origin by the World Health Organization in 2016[13] because more than 55% of cases contained the *BRAFV600E* mutation.[14,15] Thus, *BRAF* mutational status can be used to confirm the diagnosis. Mutations in *NRAS*, *ARAS*, *KRAS*, *PIK3CA*, *MAP2K2*, *AKT1*, and other genes involved in the MAPK activation pathway are also common. Furthermore, *BRAF* pseudogene overexpression occurs in various tumors, particularly diffuse large B-cell lymphoma, where they regulate the parental *BRAF* gene posttranscriptionally and contribute to cancer development.[16]

In this study, we examined the expression of *BRAF* gene and *BRAF* pseudogene in 2 Japanese patients with ECD, with or without the *BRAFV600E* mutation.

2 | METHODS

2.1 | Participants

One of the 2 patients we evaluated (ECD1, reported previously[12]) was a 32-year-old man with exophthalmos, diabetes

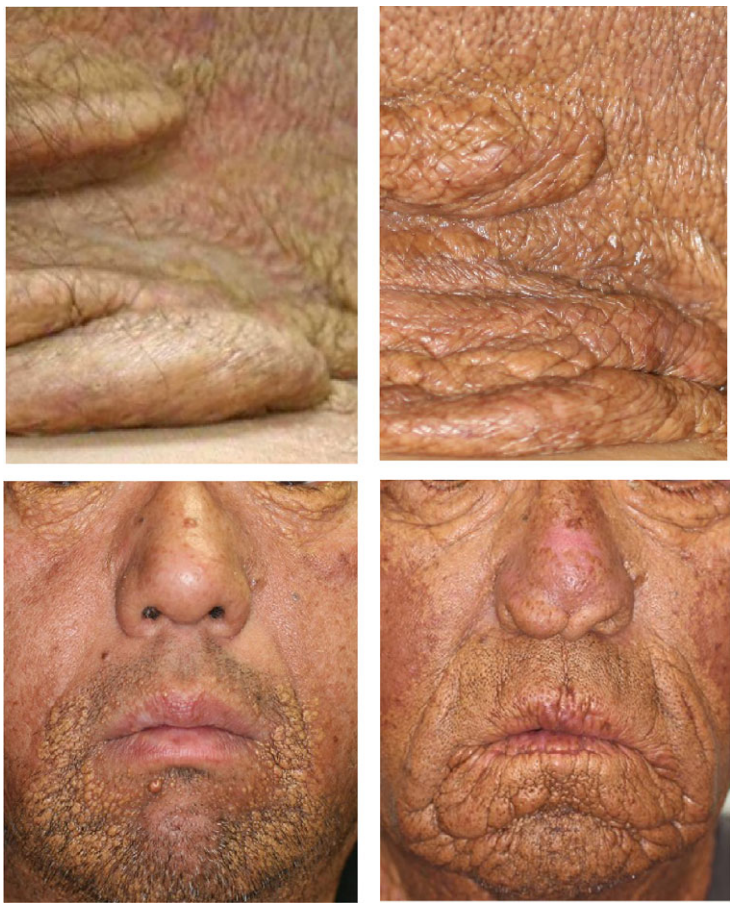

FIGURE 1 Photographs of patients ECD1 (neck, upper views) and ECD2 (lower eyelid/mouth, lower views) showing initial (left) and biopsy (right) presentations. Xanthomas of the neck in patient ECD1 became atrophic, whereas those of patient ECD2 expanded

insipidus, and multiple xanthomas of the periorbital, neck, and flexor extremity skin. The other patient (ECD2) was a 44-year-old man with multiple xanthomas of the eyelid, neck, and flexor extremity skin (Figure 1). No other abnormalities were observed. Four years later, however, the xanthomas appeared bulkier, and additional sites of involvement (nasopharyngeal xanthomas, myocardial infiltration, perinephric infiltration, and bony sclerosis) were observed.

TABLE 1 Primers used for amplification of *BRAF*-specific cDNA, genomic *BRAF*, *BRAF* pseudogene, 18S rRNA, and beta-actin

	Primers	
Gene	Direction	Sequence 5′→3′[a]
BRAF specific		
speS	Sense	TCCGGAGGAGGTG**TGGAATAT**
speAS	Antisense	**TTG**A**AAAAACTGAAAGAGA**TG**AAG**GTAGC
BRAF genome		
gS[b]	Sense	TCATAATGCTTGCTCTGATAGGA
gAS[b]	Antisense	CTAGTAACTCAGCAGCATCTC
BRAF pseudogene		
pS1	Sense	ATCCTGCCATTCCCGTGG**AG**
pS2	Sense	GAAAAGAAA**GA**A**CC**A**G**TAGGA**CA**AATACC
pAS	Antisense	**GGAAAACTGAAAGAGAT**A**AAGGT**TTTTTG AAAAAC
18S rRNA	Sense	CGATGCTCTTAGCTGAGTGT
	Antisense	GGTCCAAGAATTTCACCTCT
Actin	Sense	AGAGCTACGAGCTGCCTGAC
	Antisense	AGCACTGTGTTGGCGTACAG

[a]The sequences common to BRAF gene and BRAF pseudogene are highlighted in bold.
[b]These primers are from Ref. 15.

2.2 | Specimens of skin tissue and PC3 cell line

All skin samples were obtained at surgery following ethics committee approval and informed consent. Samples were stored at −80°C until processed for gene analysis. The patient specimens included a right occipital xanthoma and normal tissue adjacent to a right cervical xanthoma from patient ECD1 and a right inferior palpebral xanthoma and normal tissue from the right axilla of patient ECD2. Normal human skin (NH1) from the back was obtained from a 52-year-old healthy man. The PC3 cell line (ID: TKG 0600) overexpressing *BRAF* gene and *BRAF* pseudogene[16] was obtained from the Tohoku University Cell Resource Center for Biomedical Research.

2.3 | Tissue fixation, hematoxylin and eosin staining, and immunohistochemical staining

Tissues were fixed in 4% paraformaldehyde overnight and embedded in paraffin according to standard procedures. We stained 4 μm sections with hematoxylin and eosin, CD68 (M0814; Dako), or CD163 (bs-2527R; Bioss).

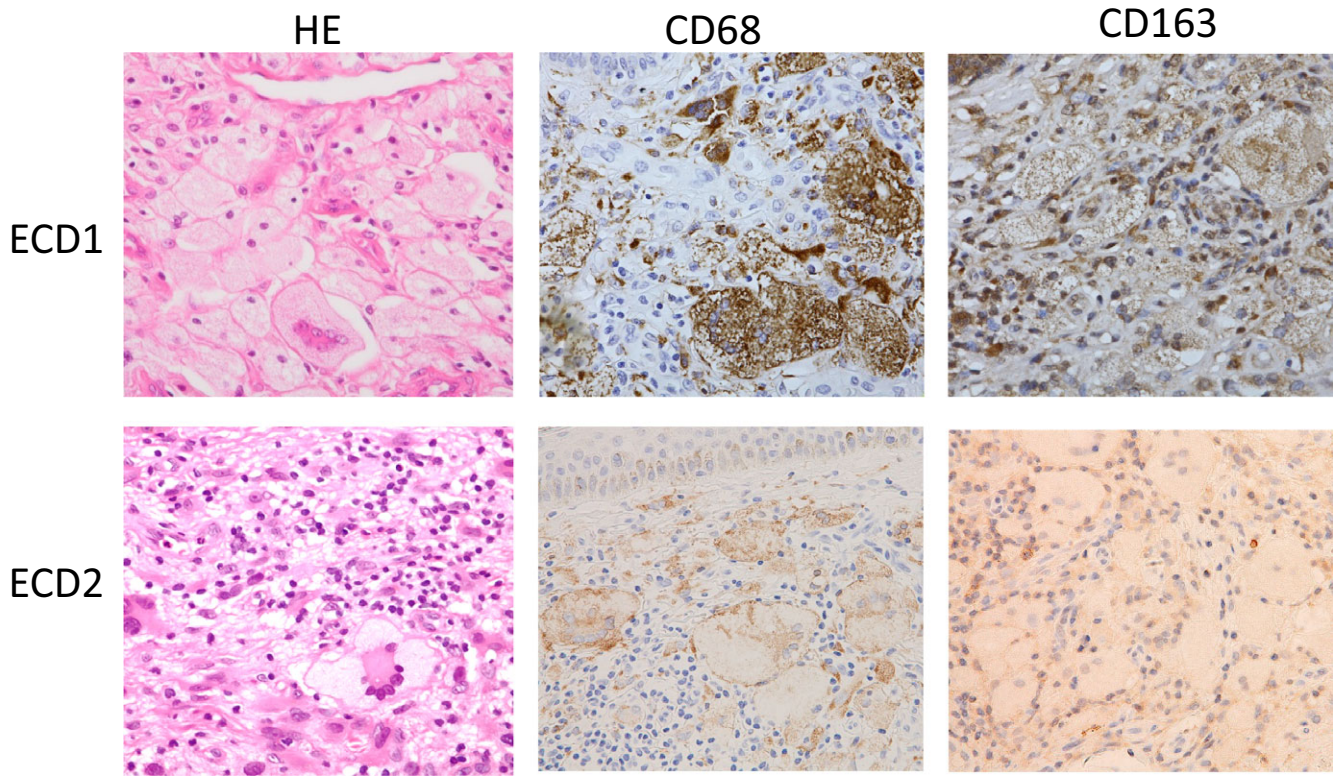

FIGURE 2 Histologic features of xanthomas from patients ECD1 (upper) and ECD2 (lower) showing hematoxylin and eosin staining (left) and immunostaining with CD68 (middle) and CD163 (right). Magnification: 40×

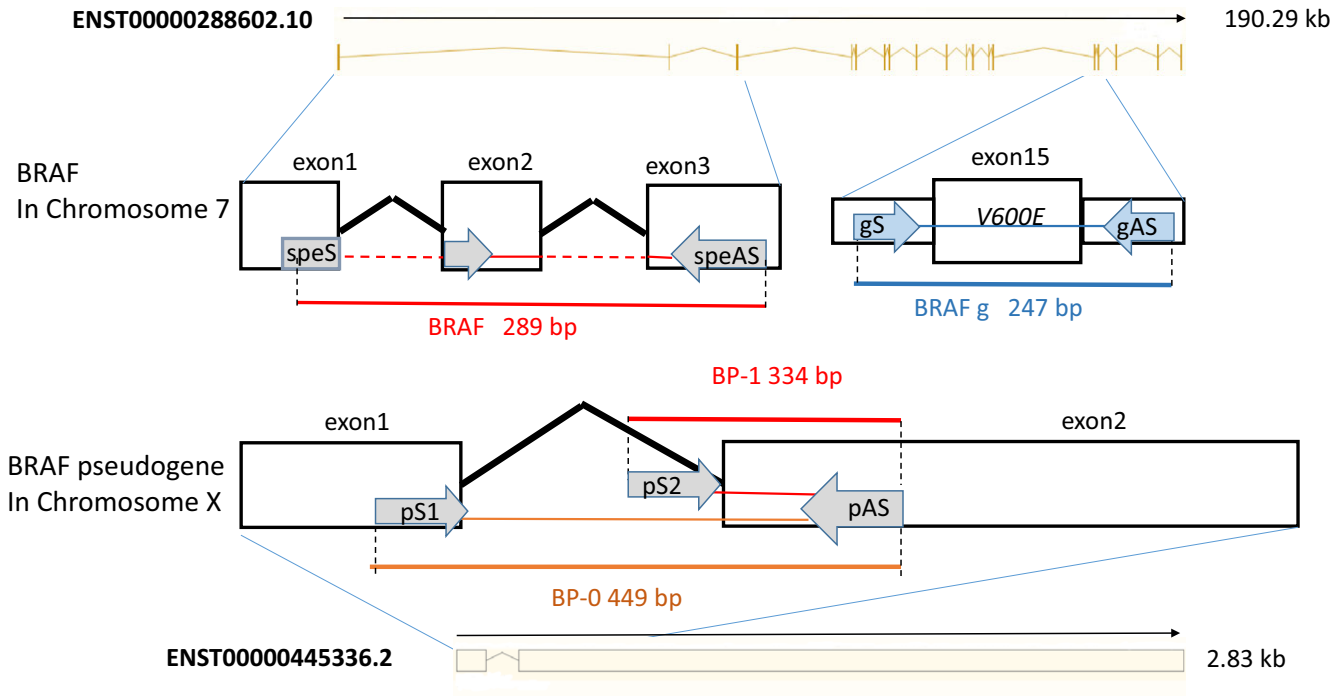

FIGURE 3 Schema of *BRAF* and *BRAFP1*. The intron sits between exon 1 and exon 2 on the *BRAF* pseudogene by ENST00000445336.2, with no splicing

2.4 | DNA amplification and sequencing analysis of exon 15 of *BRAF*

Genomic DNA of xanthoma tissue was extracted using NucleoSpin DNA RapidLyse (TaKaRa) according to standard procedures. Exon 15 was amplified by the polymerase chain reaction (PCR) using primers gS and gAS (Figure 3, Table 1)[17] and KODFX (TOYOBO). The PCR conditions were 94°C for 2 minutes, followed by 36 cycles of amplification (98°C for 30 seconds, 56°C for 30 seconds, 68°C for 30 seconds), and finally 68°C for 3 minutes. After purification of PCR products using LaboPass PCR (Cosmo Gene Tech), the amplified fragment was directly sequenced using BigDye Terminator v3.1.

2.5 | Reverse transcription PCR and real-time PCR

Total RNA was isolated using Maxwell 16 LEV simplyRNA Tissue Kit. RNA concentration was measured with the NanoDrop 2000 spectrophotometer, and RNA integrity was verified on 2% denaturing agarose gels. ReverTra Ace qPCR RT Master Mix (TOYOBO) was used for RNA reverse transcription. The reaction was conducted in a 10 μL mixture.

Total RNA was reverse-transcribed, and real-time PCR was performed to amplify cDNA via primer sets specific for either *BRAF* or *BRAF* pseudogenes—speS, speAS, pS1, pS2, and pAS (Figure 3, Table 1)[17]—using THUNDERBIRD SYBR qPCR Mix (TOYOBO) and the QuantStudio 7 Real-Time PCR machine. The PCR conditions were as follows: 95°C for 1 minute, followed by 38 cycles of amplification (95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 1 minute), and finally 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds.

3 | RESULTS

3.1 | Cells of xanthomas were macrophages showing CD68 and CD163 positivity

Histological examination of the xanthomas from the patients showed foamy macrophages and Touton-type giant cells. Immunohistochemical staining of them revealed CD68 and CD163 positivity (Figure 2), thus providing an ECD diagnosis according to the stipulated guidelines.[2]

3.2 | *BRAFV600E* mutation was found in genomic DNA from ECD1 but not ECD2

Because patients with ECD have *BRAF* mutations, screening for likely V600E point mutations was performed. Genomic DNA encoding exon 15 (Figure 3, Table 1)[17] was amplified in both patients. The *BRAFV600E* mutation was detected in ECD1, but not in ECD2, during sequence analysis (Figure 4).

3.3 | ECD1 and ECD2 exhibited overexpression of *BRAF* and *BRAF* pseudogenes compared with normal tissues

No genomic contamination was evident, as no amplification was observed when using mRNA as a template. *BRAF* pseudogene expression by PC3 cells was approximately 1000 times lower than *BRAF* expression using the standard curve method. Both BP-0 and BP-1 *BRAF* pseudogenes were detected in cDNA of patients with

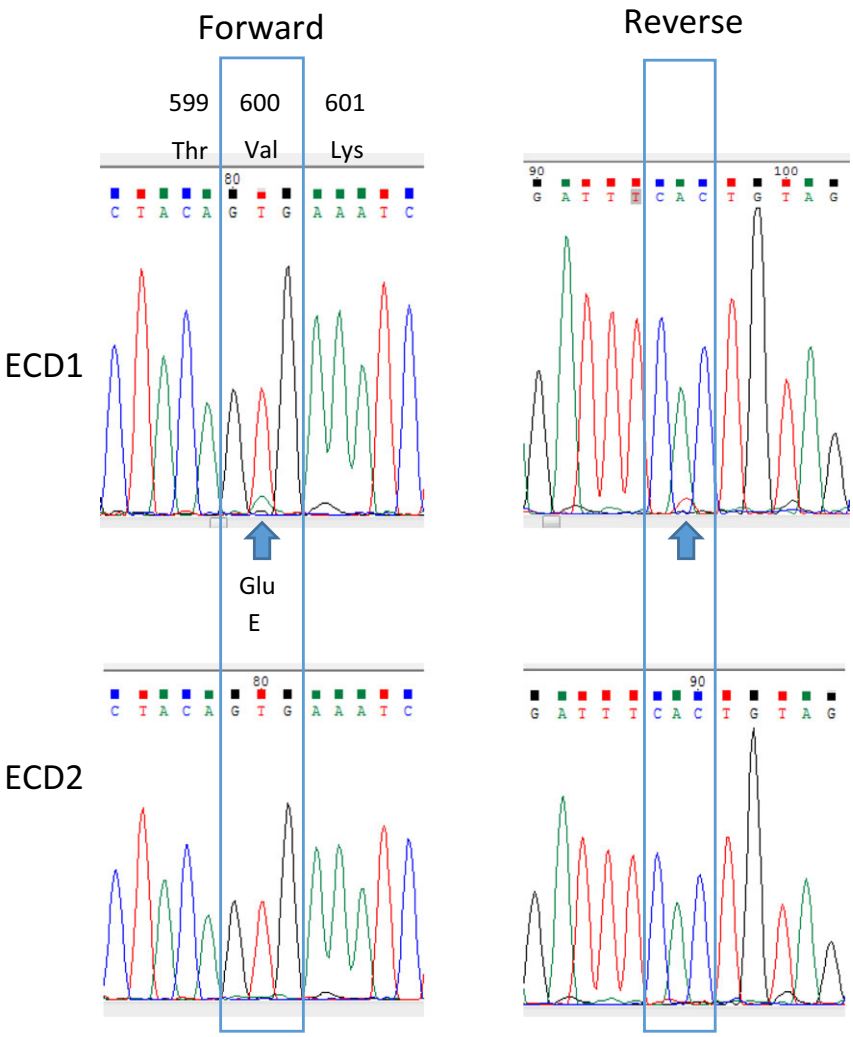

FIGURE 4 Sanger sequencing of PCR products from genomic DNA isolated from xanthomas (blue squares indicate *BRAFV600* bases)

ECD and in the positive control (PC3 cells), but not in NH1 normal skin. *BRAF* genes were amplified in patients and control samples (Figure 5). The PCR products using BP-0 primers were 449 base pairs (bp) and using BP-1 primers were 334 bp, including the intron. Thus, there was no splicing out of the *BRAF* pseudogene, as previously reported,[16,17] and no mutation in the PCR products based on sequencing analyses. Expression levels of both *BRAF* and *BRAF* pseudogenes were higher in xanthomas than in normal tissues (Figure 5).

4 | DISCUSSION

The macrophages in our ECD cases showed positivity of CD163, which shows M2-like polarized tumor-associated macrophages promoting cancer cell growth.[18] Recent studies demonstrate that many patients with ECD have mutations in *BRAF*, *NRAS*, and other genes involved in the MAPK activation pathway (similar to lymphoma), leading to categorization of ECD as a lymphoid neoplasm.[13] Mutated BRAF proteins have activated serine/threonine kinase activity, which accelerates the RAF-MEH-ERK-MAP pathway and induces cell growth.[19]

Pseudogenes are subclasses of long noncoding RNAs derived from protein-coding genes. Although not capable of producing proteins, they show high sequence homology with their protein-coding parental counterparts and regulate parental genes posttranscriptionally through common microRNAs that act as competitive endogenous RNAs. As with other pseudogenes, *BRAF* pseudogenes are overexpressed in various tumor types, including diffuse large B-cell lymphoma, melanoma, prostate cancer, and lung cancer cell lines, thus contributing to the development of cancer.[16]

Both patients reported herein exhibited elevated levels of *BRAF* and *BRAF* pseudogenes, with the *BRAFV600E* mutation found in only patient ECD1. The xanthomas of patient ECD1 became atrophic after partial extirpation (Figure 1), and no new infiltration of other organs materialized in the subsequent 10 years. Patient ECD1 has been stable since 2006, although at an advanced stage of the disease. Patient ECD2 had active early disease upon diagnosis. In a few months prior to diagnosis, he developed knee pain, chest discomfort, and hoarseness, which led to the diagnosis of ECD.

The *BRAFV600E* mutation is detected more often in advanced stages of thyroid cancer than in early-stage disease, whereas *BRAF*

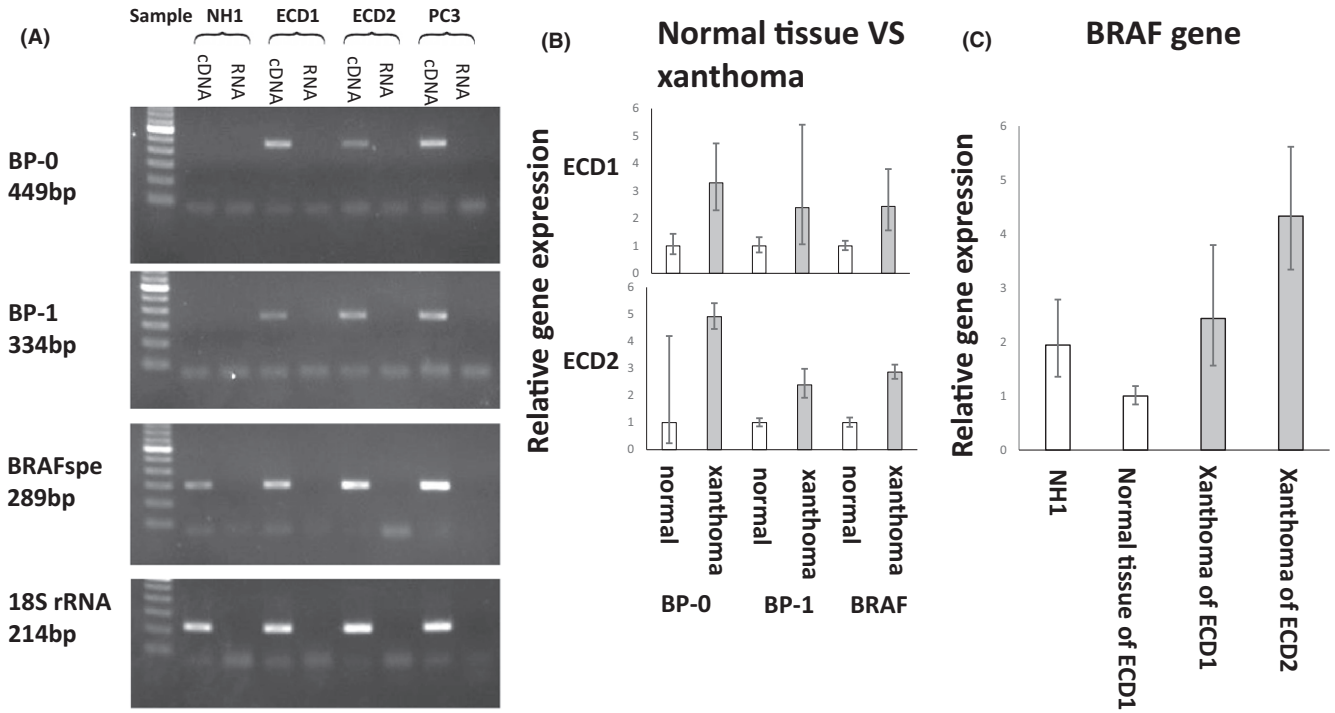

FIGURE 5 *BRAF* pseudogene expression in normal human skin (NH1), xanthomas of patients with Erdheim-Chester disease (ECD), and the PC3 cell line (positive control). The cDNA and mRNA from NH1, ECD1, ECD2, and PC3 samples indicate no genomic DNA contamination (A). *BRAF* and *BRAF* pseudogene expression levels in normal skin and xanthomas of patients ECD1 and ECD2. BP-0 (left), BP-1 (middle), and BRAF (right). Normal tissue: white bars; xanthoma: gray bars (B). *BRAF* expression levels in normal skin (NH1), uninvolved tissue from patient ECD1, and xanthomas from patients ECD1 and ECD2 (C)

pseudogene expression is heightened initially, but lower in advanced stages.[17] Similarly, the *BRAFV600E* mutation was associated with advanced stage in patient ECD1, who had completed the development of cutaneous xanthomas; conversely, the mutation was lacking in patient ECD2, who exhibited early-stage disease. However, high levels of *BRAF* pseudogene expression in patient ECD2 may boost *BRAF* expression, promoting the proliferation of xanthomas.

The FDA-approved BRAF inhibitor vemurafenib has been commercially available for treating ECD patients with *BRAFV600* mutations since November 6, 2017. Favorable outcomes have been reported in a number of patients receiving vemurafenib for ECD.[2,20–22] Franconieri et al reported an ECD patient without a *BRAFV600E* mutation initially, but 11 months later, the patient developed pericarditis and 20% of the pathologic cells contained the *BRAFV600E* mutation. Vemurafenib was effective for this case of active ECD.[23] It is also possible that in the absence of the *BRAFV600E* mutation, those patients showing elevated *BRAF* expression may benefit from this treatment as well.

Herein, we profiled 2 patients with ECD, both showing heightened expression of *BRAF* gene and *BRAF* pseudogenes, with or without the *BRAFV600E* mutation. Aside from *BRAF* mutation status, expression levels of *BRAF* and *BRAF* pseudogenes will likely help identify patients with ECD who are candidates for vemurafenib. We examined *NRAS-61* mRNA levels in xanthomas from both patients but found no mutations. It is unclear whether mutations in other genes play a role in these cases. The etiologic ramifications of *BRAF* or *BRAF* pseudogenes in patients with ECD will be the focus of future study.

APPROVAL OF THE RESEARCH PROTOCOL

The protocol for this research project has been approved by a suitably constituted ethics committee of the institution, and it conforms to the provisions of the Declaration of Helsinki.

DECLARATION

Informed consent: Participants have given their written consent for this study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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