

Germline E-Cadherin mutations in familial lobular breast cancer

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Abstract

Background: The cell surface glycoprotein, E-Cadherin (*CDH1*) is a key regulator of adhesive properties in epithelial cells. Germline mutations in *CDH1* are well-established as the defects underlying the Hereditary Diffuse Gastric Cancer (HDGC) syndrome: an increased risk of lobular breast cancer (LBC) has been described in HDGC kindreds. However, germline *CDH1* mutations have not been described in LBC patients outside of HDGC families. We sought to investigate the frequency of germline *CDH1* mutations in LBC patients with early onset disease or family histories of breast cancer without DGC.

Methods: Germline DNA was analyzed in 23 women with invasive lobular or mixed ductal and lobular breast cancers who had at least one close relative with breast cancer or had themselves been diagnosed before age 45, had tested negative for a germline *BRCA1* or *BRCA2* mutation, and reported no personal or family history of diffuse gastric cancer. The full coding sequence of *CDH1* including splice junctions was PCR amplified and screened for mutations using DHPLC and sequencing.

Results: A novel germline *CDH1* truncating mutation in the extracellular portion of the protein (517insA) was identified in one subject who had lobular breast cancer at age 42 and a first degree relative with invasive lobular breast cancer.

Conclusions: Germline *CDH1* mutations can be associated with invasive lobular breast cancer in the absence of diffuse gastric cancer. The finding, if confirmed, may have implications for management of individuals at risk for this breast cancer subtype, and compels clarification of the cancer risks in the syndrome.

Keywords: Lobular Breast Cancer, germline mutations, *CDH1*, familial breast cancer

Key points:

- Germline *CDH1* mutation was found in a woman with Lobular Breast Cancer (LBC) and family history of breast cancer but not diffuse gastric cancer.
- LOH was demonstrated in tumor specimen from the mutation carrier.
- These results, if confirmed, have implications for the genetic basis of LBC and for the identification and management of individuals at risk.

Introduction

The existence of a strong hereditary predisposition to breast cancer has been recognized for more than a century. Germline mutations in *BRCA1* and *BRCA2* have been shown to account for approximately one-third of hereditary breast cancers, among young women with the disease. Mutations in other genes such as *TP53*, *PTEN*, *STK11*, *CHEK2* and *ATM* account for a small proportion of hereditary breast cancer syndromes, often with distinct clinical features¹. However, in many breast cancer families, no predisposing gene mutation can be identified. Although the existence of other strongly predisposing genes is controversial, the search for additional breast cancer susceptibility genes remains an active area of investigation.

The *CDH1* (epithelial cadherin, OMIM 192090) gene is composed of 16 exons located on chromosome 16q22.1². The calcium-dependent molecule E-cadherin, a key regulator of cell adhesion, is the protein product of *CDH1*³, and is commonly used in the immunohistochemical evaluation of breast cancers, discriminating between lobular and ductal histologies. Germline inactivating mutations in the *CDH1* account for one third of hereditary diffuse gastric cancer (HDGC) kindreds⁴, defined as having two or more cases of DGC in first degree relatives, with at least one documented case of DGC before age 50 or, multiple cases of gastric cancer of which at least one is confirmed as DGC before age 50⁵⁻⁷. Germline *CDH1* mutations are inherited in an autosomal dominant manner, and are highly penetrant, conferring a cumulative risk of DGC of 67% in men and 83% in women⁵. Recently, an excess of invasive lobular (including mixed ductal and lobular histology) breast cancers has been reported in families with HDGC⁵⁻⁸. Like DGC, LBC show histologic features consistent with loss of cell-to-cell adhesiveness, and the absence of E-cadherin by immunohistochemical techniques in a substantial majority^{9, 10}. Moreover, as sporadic DGC, more than 50% of sporadic infiltrating LBC harbour inactivating somatic *CDH1* mutations accompanied by loss of heterozygosity¹¹. We identified probands with invasive lobular or mixed ductal and lobular breast cancer and either early age at diagnosis or family history of breast cancer systematically from a breast cancer databank, and analyzed their peripheral lymphocyte DNA to assess possible germline mutations in the *CDH1* gene.

Material and Methods

A group of subjects from the Dana Farber Cancer Institute were retrospectively identified from among women with breast cancer who had provided signed informed consent for an Institutional Review Board-approved banking protocol. The consent permitted collection, storage and analysis for research of medical records, peripheral blood and tumor specimens. Participants also completed a risk factor questionnaire including unconfirmed family cancer history information stored in a linked database. Specimens were stored in the annotated Dana-Farber/Harvard Cancer Center SPORE CORE Laboratory Blood Repository, which has been maintained since 2000. The criteria for the identification of the index cases were established at the beginning of the collection and included subjects who had documented invasive lobular or mixed ductal and lobular breast cancer at any age, no reported relatives with gastric tumors and either (1) family history with two or more cases of breast cancer in first or second degree relatives in the maternal and paternal lineage, including third degree relatives in the paternal lineage; or (2) lobular or mixed breast cancer diagnosed in the proband before 45 years of age independent of family history (**Table 1**). Because lobular breast cancers are observed in carriers of germline *BRCA1* and *BRCA2* mutations, the cohort was restricted to women whose germline *BRCA1/2* status was known. Those with germline mutations in the *BRCA1/2* breast cancer susceptibility genes were excluded from the analysis. *BRCA1/2* rearrangement analyses (BART™, Myriad Genetic Laboratories, Salt Lake City, UT) had been performed clinically on only 2 probands: however, the prevalence of BART-detected mutations is no more than 3% in “severely-affected” kindreds (R. Wenstrup, Myriad Genetics, personal communication) so was not performed on our cohort.

Three hundred thirty women with invasive lobular or mixed ductal and lobular breast cancer were identified from the data bank, which has enrolled more than 2000 newly diagnosed breast cancer patients at Dana Farber Cancer Institute since 1999. Family history information provided by the patient at enrollment was available in more than 90% of cases, but could not be directly confirmed under the terms of the protocol which precluded further patient contact. Forty-eight of these women had had DNA analyzed for *BRCA1* or *BRCA2* germline mutations, identified in clinical testing or in the course of other research. Among these, five were excluded because of a positive *BRCA1* (n=2) or *BRCA2* (n=3) mutation identified by sequence analysis in the patient or close relatives. Among the forty-three subjects meeting histologic criteria who had tested negative for *BRCA1* and *BRCA2* mutations, nine subjects were excluded because there was no blood specimen for DNA extraction available from the core laboratory, and 11 were excluded because of failure to meet age or family history criteria. Therefore, the analysis was limited to 23 women with documented invasive lobular (9) or mixed ductal and lobular (14) breast cancers who had previously tested negative for germline *BRCA1* and *BRCA2* mutations (**Figure 1**). Nineteen of these women met the first eligibility criterion; four women met the second. The median age at breast cancer diagnosis was 45 years (range 36-66 years) for the entire group, 46 years (range 36-66 years) for women meeting the first criterion and 40.5 (range 36-42 years) years for those in the age-related category. Medical record documentation of histopathology was assembled. Family history was confirmed when it was possible with medical records. All pathology slides were reviewed at time of clinical evaluation at the Brigham and

Women’s Hospital. After the cohort was finalized and clinical information linked to specimens, all identifiers were removed, in accordance with protocol stipulations. Genomic DNA was extracted from blood samples in the Dana Farber/Harvard Cancer Center Breast Cancer SPORE core laboratory at Dana Farber Cancer Institute using a Qiamp DNA Blood Midi kit (Qiagen, Valencia, CA). Analyses detailed below were performed at the Centre for Translational and Applied Genomics at the British Columbia Cancer Agency, Vancouver, B.C. (DH, director).

Table 1

Study criteria of subject’s inclusion for E-cadherin mutation analysis.

| Criteria for Inclusion | Cases (N) | Median Age |
|---|-----------|-----------------------|
| Proband with documented invasive lobular or mixed ductal and lobular breast cancers tested negative for germline <i>BRCA1</i> and <i>BRCA2</i> mutations and has: | | |
| 1. Family history with two or more cases of breast cancer in first or second degree relatives in the maternal or paternal lineage, including third degree relatives in the paternal lineage; or | 19 | 46 (range 36-66) |
| 2. Proband diagnosed with lobular or mixed breast cancer before 45 years of age independent of family history. | 4 | 40.5 (range 36-42) |

CDH1 analysis

Mutational Analysis:

Samples with insufficient DNA for complete mutational analysis of *CDH1* underwent whole genome amplification using the GenomiPhi DNA amplification kit (Amersham Biosciences) according to the manufacturer’s instructions. Briefly, 10 ng of DNA (10ng/uL) was mixed with 9uL of sample buffer containing random hexamer primers and heated to 95°C for 3min. After cooling, 9uL of reaction buffer and 1uL of enzyme (Phi29 DNA polymerase) was added to the sample and incubated at 30°C for 18hrs. The sample was then heated to 65°C for 10 min. to inactivate the enzyme. Amplified DNA was purified by ethanol precipitation prior to DHPLC (denaturing high performance liquid chromatography) analysis. The full coding sequence of *CDH1* including splice junctions was PCR amplified and screened for mutations using DHPLC. Primer sequences and conditions are as previously described⁷.

PCR products, which had shown a potential variant with DHPLC, were sequenced in both directions starting from a fresh PCR product. Prior to sequencing, the PCR products were purified using the Qiagen MinElute gel extraction kit (Qiagen, Mississauga, ON). Sequencing was then performed using the Big Dye Terminator V3.1 cycle sequencing kit and analyzed using the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City CA).

***CDH1* promoter methylation analysis**

CDH1 promoter methylation analysis was performed in microdissected tumor material from the proband. DNA was extracted using the Invisorb Spin Tissue Mini Kit (Invitex) following manufacturer's instructions. Approximately 200ng of DNA were treated with EpiTect Bisulfite Kit (Qiagen). Unmethylated Cytosines were converted to Uracil while methylated ones remained unmodified. Bisulfite treated DNA from white blood cells was *in vitro* methylated with M.SssI DNA MeTase and used as a positive control for methylation determination. The *CDH1* promoter CpG island 3 was PCR-amplified using flanking primers (sequences available upon request), specifically designed for bisulfite treated DNA sequences without CpG sites, and sequenced for methylation status determination.

LOH analysis

Loss of Heterozygosity (LOH) analysis was performed using microdissected tumor and DNA extracted from adjacent normal tissue.

The *CDH1* promoter common polymorphism -160C/A as well as the *CDH1* exon 4 mutation site were used as intragenic markers for LOH analysis in DNA extracted from tumor and normal material from the proband. Moreover, DNA was PCR amplified and sequenced for each site with the aim of determining whether the wild-type allele was under-represented in tumor DNA when compared to the sequencing profiles obtained from normal breast epithelia and constitutional DNA.

Results

Among 23 women with documented invasive lobular or mixed ductal and lobular breast cancer who had previously tested negative for germline *BRCA1* and *BRCA2* mutations, one novel mutation in the *CDHI* gene was detected by DHPLC and confirmed by direct sequencing (**Figure. 2B**). The 517insA mutation is located near the 5' end of the *CDHI* gene, and results in a premature stop codon, eliminating all of the transmembrane and intracellular domains and the majority of the extra-cellular domain of the protein.

The mutation 517insA was found in a woman whose LBC was diagnosed at age 42 and whose mother reportedly had lobular breast cancer at age 28. The diagnosis of the mother was confirmed with her doctor's notes. No other breast or gastric cancers were reported in the family (**Figure. 2A**). The proband's breast cancer was negative for E-cadherin (*CDHI*) by immunohistochemistry indicating that a second molecular event, towards the complete inactivation of the *CDHI* gene, had occurred (**Figure. 2C, D**). We searched for the inactivation of the wild-type allele in a microdissected tumor sample from the proband. Promoter methylation analysis was performed and no methylated alleles were found (data not shown). Subsequently, LOH analysis was performed in the same tumor sample using *CDHI* distal and proximal microsatellite markers as well as intragenic markers. No loss of genetic material was found using *CDHI* flanking markers (data not shown).

The LOH using intragenic markers revealed a different scenario: the sequencing of *CDHI* -160C/A polymorphism showed equal peak heights for both alleles in tumor material from the proband, suggesting that LOH is not occurring at this specific 5'-end of the *CDHI* gene. In contrast, the sequencing analysis for *CDHI* exon 4 performed in tumor DNA shows a clear reduction of the peak heights corresponding to the wild-type when compared with the mutant allele. This reduction could not be observed in the sequencing analysis of constitutional DNA or in DNA from normal breast epithelia. This result is suggestive of LOH, downstream of the promoter region of the gene and encompasses at least exon 4.

Discussion

In this report, we describe the finding of a germline *CDHI* mutation in a woman with lobular breast cancer whose family history includes additional lobular breast cancer but no gastric cancer. Germline mutations in *CDHI* have been previously associated with marked risk of diffuse gastric cancer (67-83%), the dominant tumor in the Hereditary Diffuse Gastric Cancer syndrome⁵. Recent observations have noted an excess of invasive lobular breast cancers in some HDGC kindreds^{7, 8, 12}. The estimated cumulative lifetime risk of breast cancer in women with germline *CDHI* mutations calculated among 11 DGC families is 39%⁸. A penetrance analysis of 4 families with a founder *CDHI* mutation confirmed the increased risk for breast cancer, with a cumulative risk of breast cancer of 52% (95% CI, 29%-94%)¹³.

Previous efforts to identify germline *CDHI* mutations in familial breast cancer patients have not been very forthcoming. In a Swedish study, 19 patients with familial breast cancer whose tumors showed loss of heterozygosity at the *CDHI* locus tested negative for germline *CDHI* mutations¹⁴. The majority of the cases (10 of 19), however, were ductal carcinomas and one was medullary, a ductal subtype. Of the remainder, two were lobular and one was mixed ductal and lobular breast cancer; information on the other five tumors was not included. Since loss of E-cadherin characterizes more than 90% of lobular breast cancers and only 5-10% of ductal histologies, this distribution of histologic subtypes is unexpected¹⁵. Lei et al did not identify a germline *CDHI* mutation in 13 patients with familial LBC¹⁶. However, in this small cohort, a positive family history was not clearly defined either for degree of relation or the number of family members with breast cancer. The search for *CDHI* germline mutations in a series of 65 LCIS patients also yielded negative results¹⁷. One study has proposed that the *CDHI* missense mutation 1774G>A (A592T) is a risk factor for comedo-type carcinoma, a pathologic variant of ductal carcinoma *in situ*¹⁴. A second germline missense mutation (1876G>A (F626V)) has been reported in an individual with LBC; no family cancer history is included in the report¹⁸. The pathogenicity of these two missense mutations is not known.

The *CDHI*-encoded protein E-cadherin is a calcium dependent cell to cell adhesion glycoprotein comprised of an extracellular domain, a transmembrane region that bridges the plasma membrane, and the highly conserved cytoplasmic tail^{2, 19}. It is one of the key molecules for the establishment of the intercellular junction complex and for the adhesive properties among epithelial cells. The cytoplasmic domain of E-cadherin directs the β -catenin mediated interaction with actin cytoskeleton and p120 controls the strength of cell-to-cell adhesion by regulating cadherin stability and retention at the cell surface. It acts in a zipper-like fashion at the tight junctions of adjacent epithelial cells¹⁹. Down-regulation of *CDHI* leads to the disruption of the tissue architecture and the increase of invasive properties of the malignant cells of epithelial origin²⁰. The loss of *CDHI* expression can occur as a result of various genetic mechanisms. For example, in sporadic DGC somatic mutations target preferentially exons 7 and 9 and promoter hypermethylation account for bi-allelic silencing of *CDHI* expression in more than 50% of this type of tumors^{10, 21}, while in most sporadic LBC, *CDHI* complete silencing is

achieved by mutations scattered along the gene accompanied by either *CDHI* promoter methylation or LOH^{10, 22}.

The 517insA mutation described in this report is located near the 5' end of the *CDHI* gene. LOH at the mutation site was found in the analysis of two separate samples extracted from the proband's lobular breast cancer. In contrast to gastric cancers from germline *CDHI* mutation carriers, in which promoter methylation is the most common second hit^{21, 23}, in this case of LBC we found that the second hit is through LOH. Interestingly, in the present study LOH was not identified using *CDHI* flanking LOH markers, but using polymorphic intragenic markers, namely the mutation site in exon 4, which revealed an intragenic deletion that encompasses at least exon 4 of the *CDHI* gene. This mechanism was previously reported in a tumor from a HDGC *CDHI* mutation carrier^{21, 23}.

Our finding suggests that genetic heterogeneity may also characterize familial invasive lobular breast cancer. Lobular breast cancers comprise 9% of the breast cancer in carriers of germline *BRCA2* mutations and only 3% in *BRCA1* mutation carriers²⁴. The present report demonstrates that *CDHI* germline mutations occur in 4.3% (1/23) of lobular breast cancer probands. Therefore, if these results are confirmed in larger series *CDHI* testing may become part of the evaluation of women with lobular breast cancer, in whom features suggesting the presence of hereditary predisposition are present. In other known cancer syndromes, histopathologic information defines subsets of cancers linked to particular genes. For example, medullary thyroid cancer is associated with activating germline mutations in the *RET* proto-oncogene, but follicular thyroid cancer occurs excessively in Cowden's syndrome with germline *PTEN* mutations. Clear cell renal carcinoma is the most frequent histology observed in the Von Hippel Lindau syndrome, while papillary renal cell carcinoma is associated with germline mutations in the proto-oncogene c-MET²⁵. Among breast cancers, medullary, atypical medullary and basal-like tumors are more frequently observed in individuals with germline *BRCA1* mutations^{26, 27}. If confirmed, the association between invasive lobular breast cancer and germline *CDHI* mutations may help to guide the genetic evaluation of affected individuals and families.

The finding raises questions for the clinical management of *CDHI* carriers. Further study will be necessary to more clearly determine the penetrance of germline *CDHI* mutations, and the proper management of women with germline *CDHI* mutations. Although LBC represent only 8-14% of all breast cancers²⁸, they account for a disproportionate number of breast cancers undetectable by screening mammogram. The role of breast MRI has not been defined in this cohort. Challenges already exist in the management of the DGC risk inherent in previously identified *CDHI* mutation carriers. We have shown that several detection methods have low sensitivity for detecting early gastric cancer in HDGC patients, including endoscopy, endoscopic ultrasound, chromoendoscopy, and PET scanning, which failed to detect early DGC in all six patients one week prior to prophylactic gastrectomy^{29, 30}.

In summary, we report a novel germline *CDHI* mutation in a woman with lobular breast cancer and family history of lobular breast cancer in the absence of DGC. Additional research can now focus on reliable estimates of the mutation frequency, spectrum,

penetrance, and range of malignancies associated with germline *CDH1* mutations. Further work to identify appropriate and effective surveillance and prevention strategies for individuals at hereditary risk of lobular breast cancer with and potentially without risk of DGC will also be critical.

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Figure 1

Flow chart of study participants.

Figure 2A: Pedigree of the family displaying a new deleterious *CDH1* insertion *in* exon 4 (517insA) and family history of breast cancer; **2B:** 517insA (1C) *CDH1* mutation screening results demonstrating dHPLC results (top) and sequencing results (bottom). The dHPLC shows the wildtype sequence in black and the mutated sequence in red. Heteroduplexes that form in PCR samples having internal sequence variation display reduced column retention time relative to homoduplexes found in control samples. Mutation is indicated by arrows in sequencing results.

2C: Hematoxylin and Eosin (H and E) staining for the infiltrating lobular carcinoma; **2D:** E-cadherin stain of the invasive lobular carcinoma from the proband: the epithelium of a normal duct is E-cadherin positive whereas the tumor cells are E-cadherin negative.

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Figure 1

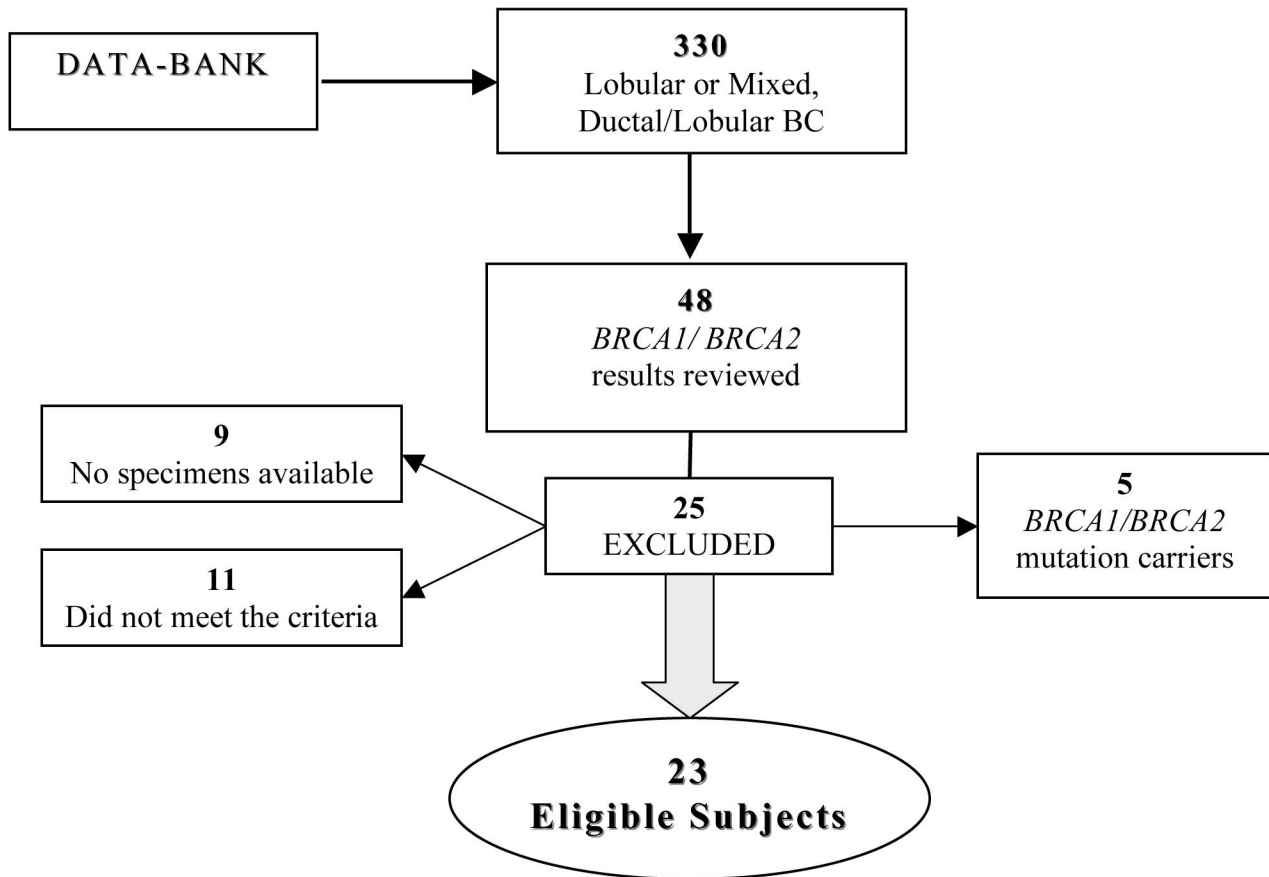
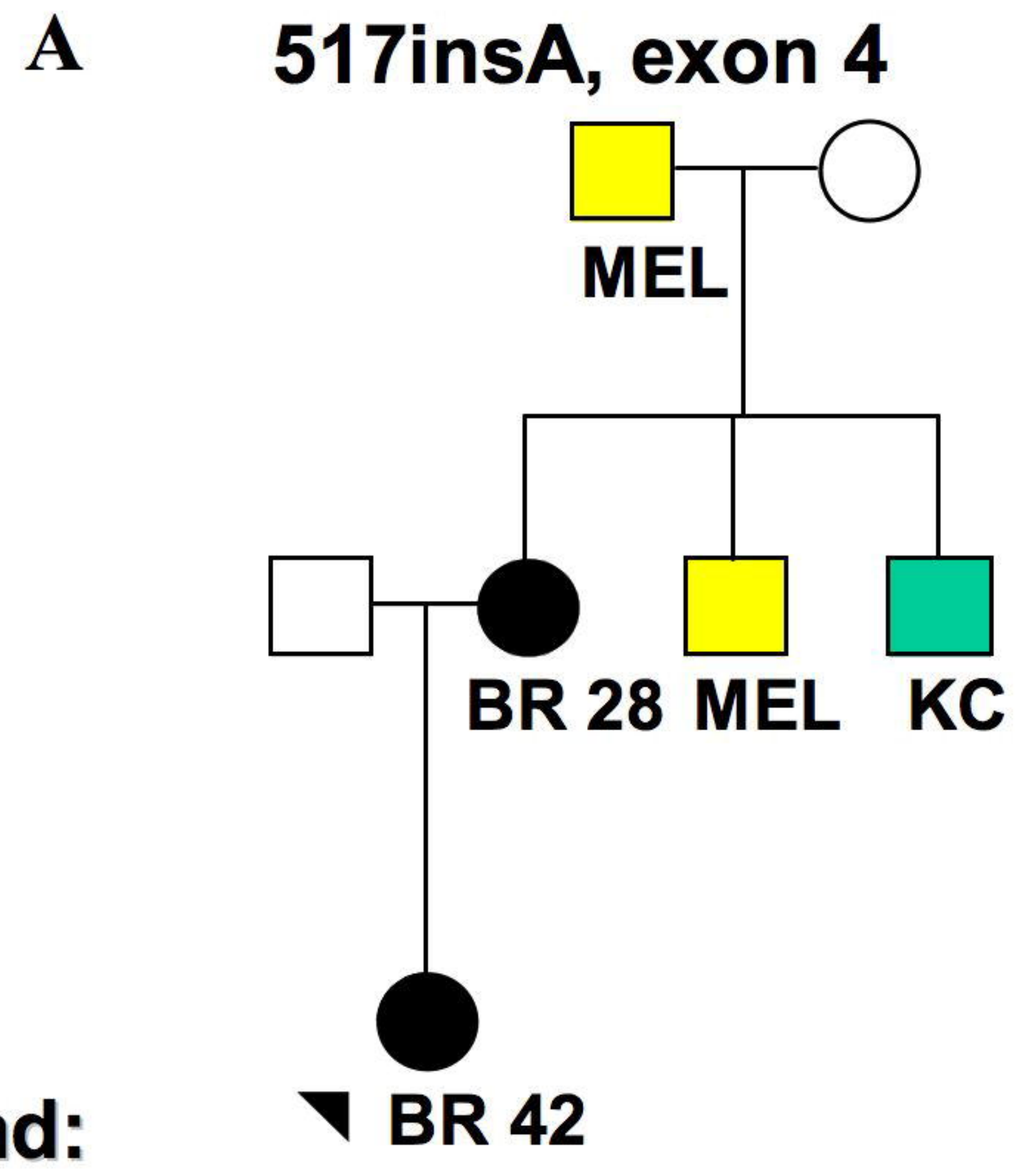
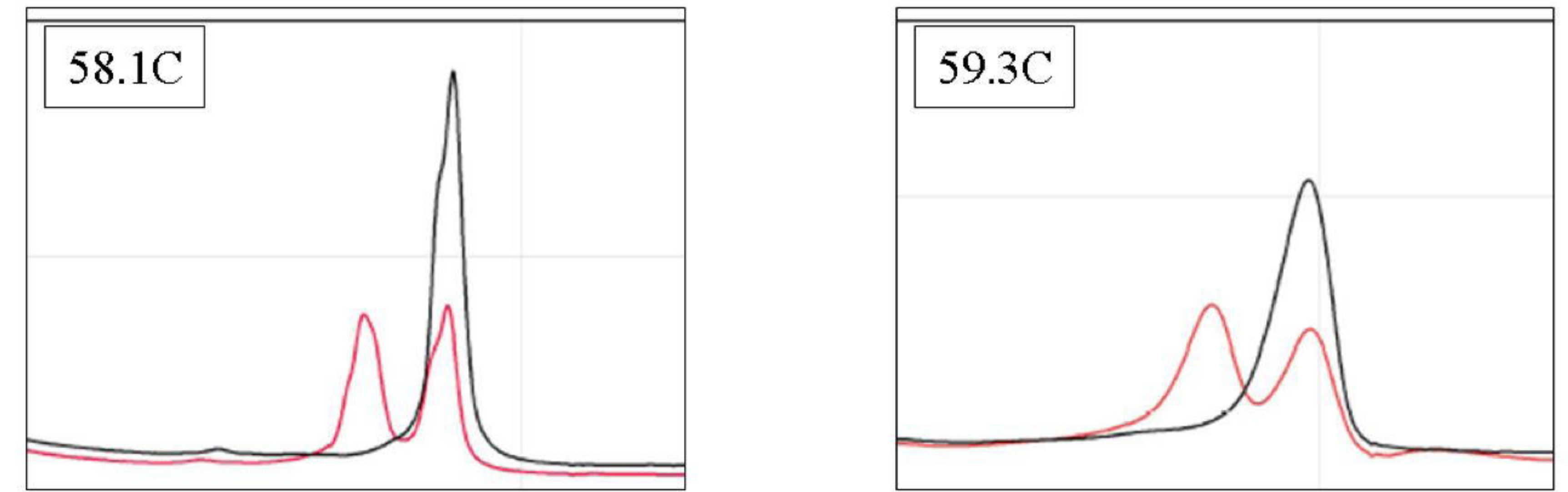


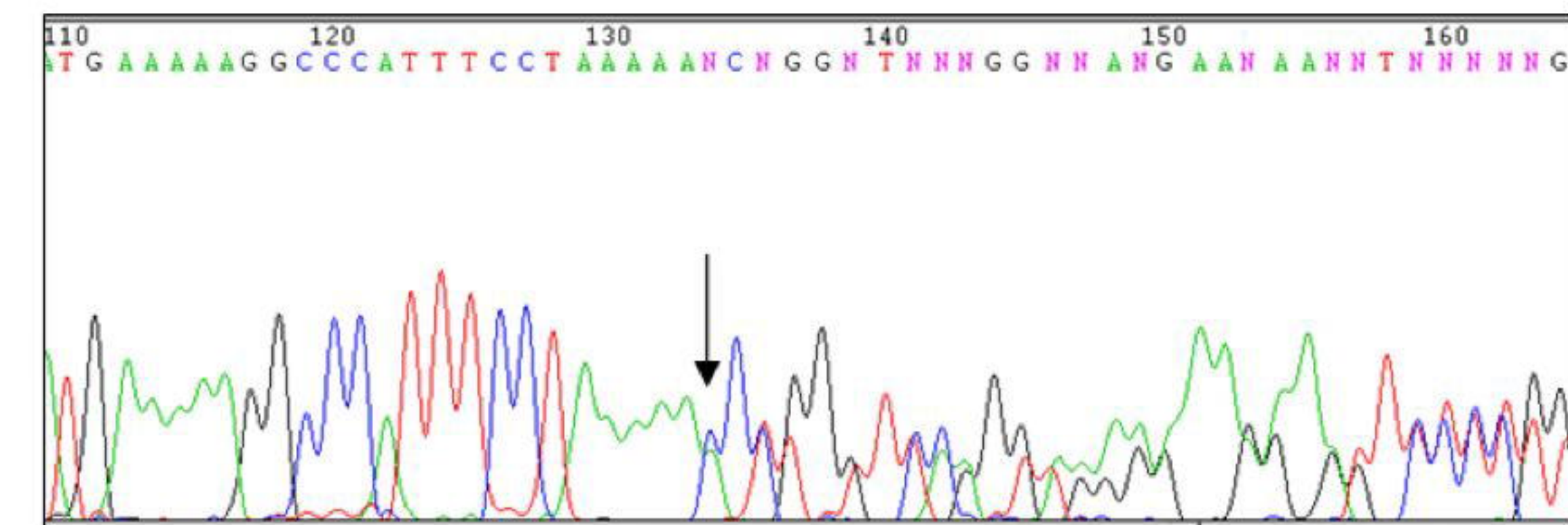
Figure 2



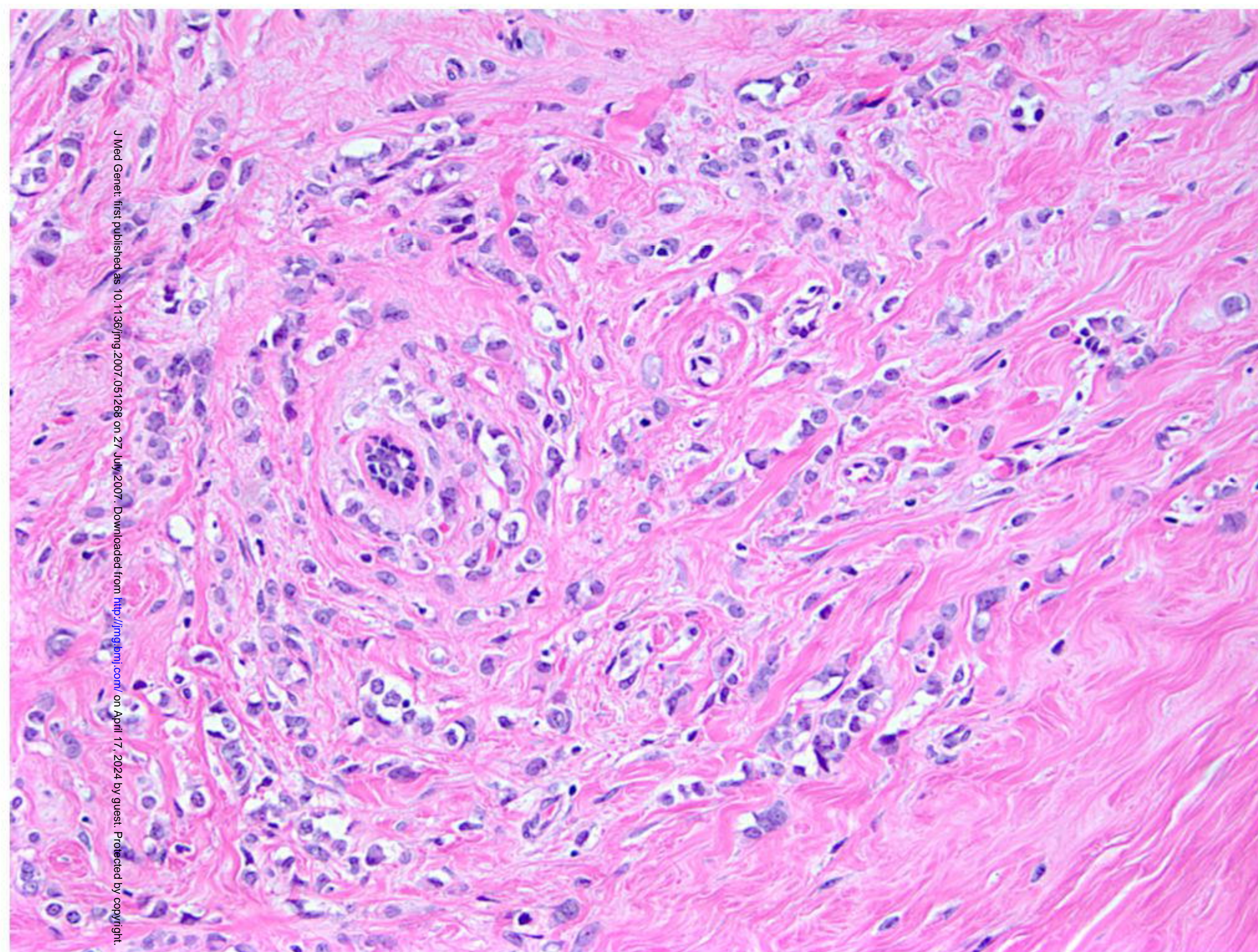
B dhplc results



sequencing results



C



D

