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A Rare Case of Embryonal Carcinoma in a Patient with Turner Syndrome without Y Chromosomal Material but Mutations in *KIT*, *AKT1*, and *ZNF358* Demonstrated Using Exome Sequencing

Claus H. Gravholt[a, b] Ole L. Dollerup[a] Lone Duval[c] Else Mejlgaard[d] Katrine Stribolt[d] Søren Vang[b] Britt E. Laursen[b, f] Michael Knudsen[b] Kasper Thorsen[b] Remko Hersmus[g] Leendert H.J. Looijenga[g] Kirstine Stochholm[a, e]

Departments of [a]Endocrinology and Internal Medicine, [b]Molecular Medicine, [c]Oncology, and [d]Pathology and [e]Center for Rare Diseases, Aarhus University Hospital, and [f]Institute of Biomedicine, Aarhus University, Aarhus, Denmark; [g]Department of Pathology, Erasmus MC-University Medical Center, Rotterdam, The Netherlands

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Abstract
Gonadoblastoma and malignant transformations thereof can occur in females with Turner syndrome (TS) and Y chromosomal material. However, in females with TS and no Y chromosomal material, this is rarely seen. We report a female with an apparent 45,X karyotype (in blood and tumor) who was diagnosed with a metastatic embryonal carcinoma. Exome sequencing of blood and the tumor was done, and no Y chromosomal material was detected, while predicted deleterious mutations in *KIT* (likely driver), *AKT1*, and *ZNF358* were identified in the tumor. The patient was treated with chemotherapy (first-line: cisplatin, etoposide, and bleomycin; second-line: paclitaxel and gemcitabine), and after that surgical debulking was performed. She is currently well and without signs of relapse. We conclude that embryonal carcinoma can apparently occur in 45,X TS without signs of Y chromosomal material.

Turner syndrome (TS) is a well-described variant of disorders of sex development (DSD) with a wide spectrum of somatic abnormalities, typically including growth retardation, gonadal insufficiency, and infertility [Gravholt, 2004]. Occurring in 50 per 100,000 liveborn with a female phenotype, it is one of the most common cytogenetic abnormalities [Gravholt et al., 1996]. The syndrome is classically associated with a 45,X karyotype, although several mosaic forms with accompanying cell lines with other cytogenetic abnormalities may be responsible. Among these, cell lines with Y chromosomal material

E-Mail karger@karger.com
www.karger.com/sxd

Claus H. Gravholt
Departments of Endocrinology and Internal Medicine and Molecular Medicine
Aarhus University Hospital
DK–8200 Aarhus (Denmark)
E-Mail ch.gravholt@dadlnet.dk

have been estimated to be present in approximately 5% of the TS population [Gravholt et al, 2000].

The presence of part of the Y chromosome has been associated with the development of gonadoblastoma, a precursor lesion with a substantial risk of malignant transformation [Tsuchiya et al., 1995]. Current recommendations suggest prophylactic gonadectomy when Y chromosomal material is demonstrated in a TS patient [Cools et al., 2006; Kanakatti et al., 2014; Gravholt et al., 2017]. A systematic search for hidden Y chromosome mosaicism with various methods has been suggested by some studies when standard karyotyping does not reveal Y chromosome positivity [Oliveira et al., 2009; Sallai et al., 2010; Cortes-Gutierrez et al., 2012].

Embryonal carcinoma is, however, a very rare event in TS without Y chromosomal material and has to our knowledge only been described once in a patient with putative 45,X [Soh et al., 1992], while a mixed dysgerminoma and embryonal carcinoma was described in another patient [Zelaya et al., 2015].

We present a case of TS with a pure 45,X karyotype, who developed a metastatic embryonal carcinoma. No Y chromosomal material was demonstrated in peripheral blood or in the tumor by exome sequencing. However, somatic mutations were identified in a selected number of genes, i.e., *KIT*, *AKT1*, and *ZNF358*.

Case Report, Methods, and Results

A 40-year-old woman with TS, treated with hormone replacement therapy (Trisequens), was otherwise healthy except for a mild, asymptomatic aortic valve stenosis. The patient was referred in May 2013 with prolonged coughing and a chest X-ray suggesting nodular infiltrates without suspicion of malignancy. She was known with a 45,X karyotype, and subsequently DNA isolated from blood was initially examined with PCR fragment length analysis for 3 Y chromosome specific markers, *SRY*, *TSPY3*, *4*, *6*, *7*, *8*, and *10* and *DYZ3* without the detection of Y chromosomal material.

Initial evaluation included a normal high resolution CT scan of the lungs and normal lung function test. Biochemical workup was inconclusive, and the case was interpreted to be most likely the result of a viral infection. Three months later, a CT of the thorax, abdomen, and pelvis revealed a cystic, pelvic tumor 16 × 9 × 18 cm, diaphragmatic metastases, a large solid metastasis in the retroperitoneum encircling the axial vessels and kidney arteries, and possibly bone metastases.

At this point the patient had complaints of a 7–8 kg unintended weight loss during a period of a few months, night sweats, light fever, and jumping pain in the columna and pelvis.

Three needle core biopsies, despite the large tumor masses, contained only small amounts of tumor cells growing in irregularly solid islands and trabeculae surrounded by inflammation and fibro-hyalinized stroma. There was no acinar pattern or amorphous hyaline material and no focal calcifications or giant cells identified. Immunohistochemical examination was performed and initially ruled out sarcoma and lymphoma (CD45 and vimentin negative). Because of the small amount of tumor in the biopsies, the color product/antibody was removed and some of the sections were restained.

Evaluating the biopsy material collectively, there was positive reaction in the tumor cells for CAM5.2 and CK8 and vague, limited positive reaction for AE1/3. There was no reaction in the tumor cells for EMA, CK7/19, CK5/6, CD30, CK20, CD45, Cdx2, Ca-125, CEA, WT-1, p63, GATA3, vimentin, RCC, estrogen receptor, mammaglobin, HEP1, S-100, CD66, chromogranin A, and synaptophysin. The proliferation index evaluated by ki-67 was high, almost 50%. Knowing that the patient had TS, an additional immuno-histochemical examination was performed and revealed positive reactions for OCT-4 (octamer-binding transcription factor), PLAP (placental alkaline phosphatase), CD117, and D2–40 and negative reactions for hCG, inhibin, and calretinin (Fig. 1).

Based on the clinical information, morphology, and the immune profile, the tumor was regarded as a malignant germinal cell tumor and most likely an embryonal carcinoma.

Tumor and normal (blood) DNA was then isolated using standard methods, and sequencing libraries were prepared using the KAPA Hyper Prep kit (KAPA Biosystems). Target enrichment was performed with the SeqCap EZ Exome v3 (Roche/Nimblegen) and paired sequenced (2 × 150 bp) with v2 chemistry on the NextSeq (Illumina). The Trim Galore program was used for trimming the adaptor sequences and then mapped to the human genome (hg19) using BWA-MEM [Li and Durbin, 2009]. During the adapter attachment in the library preparation, a number of PCR cycles were performed, which may have produced an overrepresentation of some DNA fragments. These redundant fragments were uninformative, and were removed using the Picard MarkDuplicates tool. Read mapping can have a hard time identifying insertions and deletions near the ends of the reads, and the final alignment was therefore examined by the GATK [McKenna et al., 2010] realigner and INDELs were adjusted. Each sequenced base is assigned a quality score by the sequencing machine that in some cases may need adjustment. Using several external sources of known variations, GATK BaseRecalibrator adjusts dubious quality scores to prevent over- or underestimation of called sequence variations.

The tumor was compared to a number of in-house sequenced normal samples of both genders. Raw sequence reads were counted using SAMtools [Li et al., 2009], including multi-mapping reads to deal with the pseudoautosomal regions of the sex chromosomes. All samples were counted for chromosomes X and Y as well as a set of Y-located genes and were normalized. We failed to detect any Y chromosomal sequences in DNA from blood or the tumor.

Subsequently, we compared germ line DNA (blood) with tumor DNA using GATK MuTect2 and the COSMIC and dbSNP databases as internal standards. The resulting lists of somatic variations (both SNPs and INDELs) were annotated using Oncotator [Ramos et al., 2015] and further analyzed using Ingenuity Variant Analysis (Ingenuity, Qiagen, USA).

We discovered 4 somatic nonsynonymous mutations. The analysis showed a gain-of-function mutation in *KIT* (KIT proto-oncogene receptor tyrosine kinase) (c.1965T>A; p.N655K; NM 000222.2), a gene previously shown to be involved in several can-

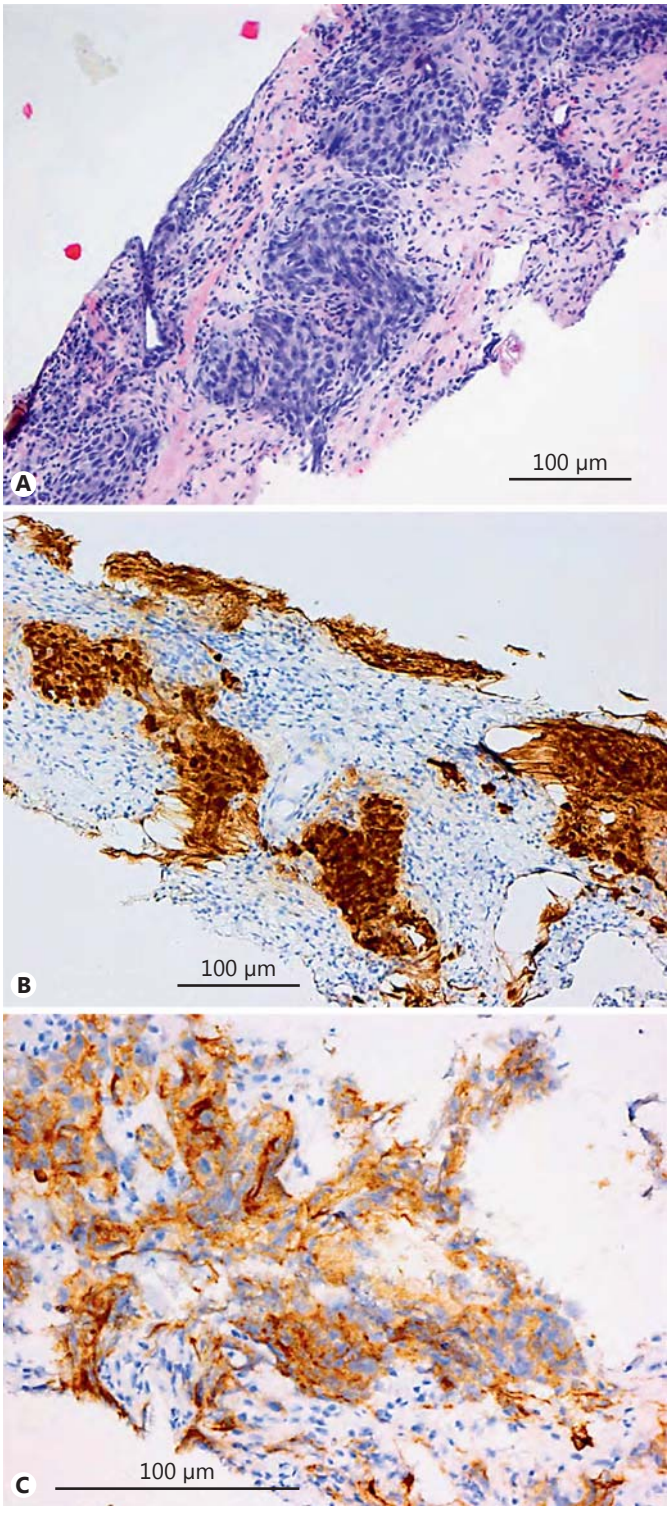

Fig. 1. Immunohistochemistry of the tumor. **A** Haematoxylin eosin staining of the tumor. **B** OCT3/4 staining of the tumor showing cytoplastic staining pattern besides the nucleus, which is supportive of the diagnosis of embryonal carcinoma [Hersmus et al., 2008]. **C** PLAP staining of the tumor showing patchy staining in the tumor. Scale bars, 100 µm.

cers and specifically in intracranial germinomas [Wang et al., 2014], testicular seminomas [Kemmer et al., 2004], as well as in gastrointestinal stromal tumors (GIST) [Corless et al., 2011] and in female patients without DSD but diagnosed with dysgerminomas [Hoei-Hansen et al., 2007; Hersmus et al., 2012]. This specific mutation has been found in GIST and melanoma [Minarik et al., 2013]. Normal expression of the *KIT* gene is important for intracellular signaling in several pathways, such as the MAPK, PI3K-AKT, and STAT3 pathways, for which the stem cell factor (SCF or KITLG) is the ligand of KIT. Based on the available literature and the analysis performed using Ingenuity, the mutation was predicted to be pathogenic.

A loss-of-function mutation in *AKT1* (v-akt murine thymoma viral oncogene homolog 1) (c.967G>A; p.D323N; NM 0051632) was also identified, and this mutation was also predicted to be damaging with a PolyPhen2 score of 0.985. AKT1 is a serine-threonine protein kinase and a member of the PI3K-AKT1-mTOR signaling cascade.

Two loss-of-function mutations were seen in *ZNF358* (zinc finger protein 358) (c.1223C>G; p.A408G and c.1225G>C; p.A409P; NM 018083.4), also involved in the MAPK pathway via the ubiquitin C protein pathway.

Analysis of copy number variations (CNV) was performed on both normal and tumor samples using the CNVkit software [Talevich et al., 2016]. We found 8 copies (normally 2 copies) in the tumor for the gene *KMT2E*, and 5 copies for *NFE2L3*, *CBX3*, and *MALAT1* (metastasis lung adenocarcinoma transcript 1). Low expression of *KMT2E* may result in poorer survival among patients with acute promyelocytic leukemia [Lucena-Araujo et al., 2014]. *MALAT1* is a long noncoding RNA (lncRNA), which has recently been shown to be involved in unfavorable survival in several types of cancer, including epithelial ovarian cancer [Zou et al., 2016]. In addition, we found several other CNVs with lower counts and of uncertain importance.

At the time of diagnosis (December 2013), the patient was referred to the Department of Oncology and immediately commenced treatment according to the poor prognosis germ cell tumor group, consisting of 3 weekly cycles of cisplatin, etoposide, and bleomycin. Side effects included bone marrow toxicity, hypomagnesaemia, and skin toxicity (rash). Evaluation by CT and MRI scans after 4 treatment cycles revealed regression which qualified, according to RECIST (www.recist.com), to a partial response. Bone metastases, despite not representing measurable disease, seemed more lytic, and the disease was therefore not considered suitable for surgical debulking nor arrested, wherefore a decision on a second-line chemotherapy was made. This regimen consisted of 3 weekly cycles of paclitaxel and gemcitabine. Evaluation was carried out after 3 and 6 series, with no change according to RECIST. Bone metastases seemed less lytic, which was interpreted as a sign of treatment benefit, yet again not representing measurable disease. The tumor biomarker human chorionic gonadotropin (hCG) instantly fell from 52×10^3 to 4×10^3 U/L after the first cycle of chemotherapy and remains below 1, while lactate dehydrogenase also rapidly declined from a maximum of 2,113 to 275 U/L at day 1 of the second treatment cycle and has subsequently remained just above or within the normal range (Fig. 2).

Follow-up, including clinical control, blood samples, and CT scans every 3 months, so far from August 2014 until August 2016, has been without evidence of disease in progression. The patient's performance status is now close to 0, while during treatment it was

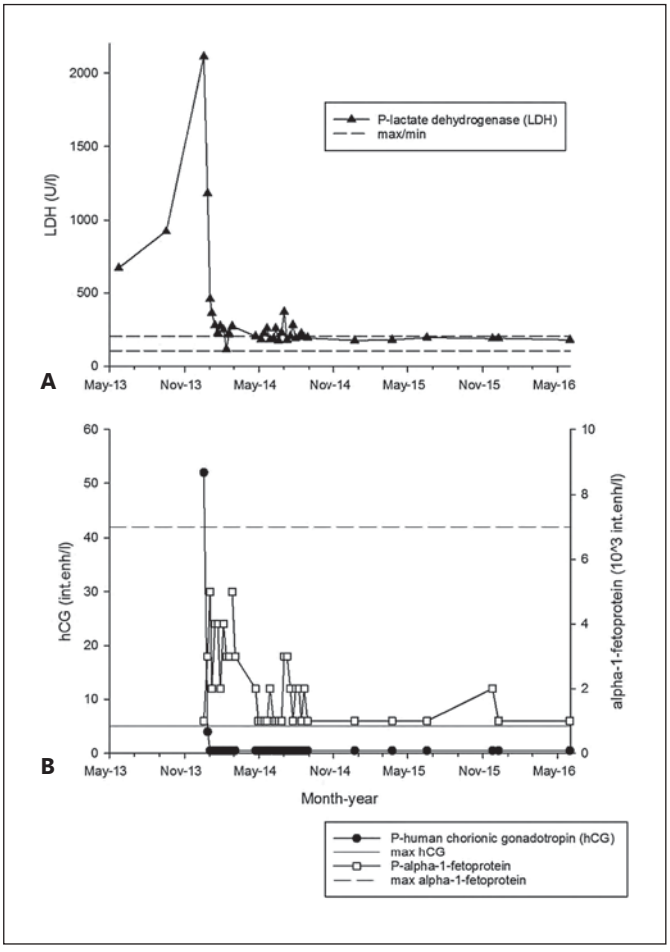

Fig. 2. Circulating levels of lactate dehydrogenase (**A**), human chorionic gonadotropin, and alpha-1-fetoprotein (**B**).

1. Subsequently, debulking was then performed because of the excellent condition of the patient. Here, the right ovary was found with tumor masses, while the left ovary could not be found, and thorough pathological examination of the surgical specimen did not reveal any malignant cells or signs of teratoma. Three years after diagnosis, the patient is well and without symptoms or signs of recurrence and is back to work.

Discussion

The most common precursor lesion of malignancy in a dysgenetic gonad in patients with TS is gonadoblastoma. A gonadoblastoma is regarded as the carcinoma in situ of the dysgenetic ovary, now referred to as germ cell neoplasia in situ (GCNIS) [Berney et al., 2016], with potential to undergo malignant transformation into invasive germ cell tumors (GCT). Most commonly, a gonadoblastoma progresses to a dysgerminoma, less frequently to tumors such as yolk sac tumor, teratoma, and embryonal carcinoma [Bai et al., 2013; Ulbright, 2014]. This seems to be related to the abdominal position of the gonad.

In the female and male population with a normal genetic constitution, pure embryonal carcinoma is rare and accounts for 4% of ovarian GCTs [Smith et al., 2006] and 2% of testicular GCTs, while being a histologic component of 85% of testicular mixed GCTs [Krag et al., 1984; Mostofi et al., 1988], because it represents the stem cell component of all other nonseminomatous elements. In both genders, it is an aggressive malignant tumor although overall responsive to chemotherapy.

Dysgerminoma is the most common malignant primitive CGT of the ovary and occurs almost exclusively in children and young women [Cools et al., 2006]. The tumor is often located in the abdominal cavity and is usually large, being >10 cm. Serum lactate dehydrogenase is often elevated, and 3–5% of patients have elevated hCG levels. Dysgerminoma is the most common malignant gonadal germ cell tumor in patients with gonadal dysgenesis and a partial or complete Y chromosome. It typically arises from a gonadoblastoma in this setting. We did consider if our case represented a dysgerminoma arising from an underlying gonadoblastoma – having in mind that no Y chromosomal material was demonstrated in peripheral blood. The morphology of the tumor and the negative stains for sex cord type cells speaks against this but does not exclude the theory.

In association with 45,X TS, embryonal carcinoma or dysgerminoma is extremely rare. A literature search only revealed 2 cases. One case is a pelvic GCT with histological findings consistent with embryonal carcinoma in a 22-year-old TS female with a 45,X chromosomal constitution [Soh et al., 1992]. The patient was unsuccessfully evaluated for Y chromosome mosaicism with standard karyotyping techniques of 90 leukocytes and a skin culture. Further investigation was not performed, leaving the possibility that mosaicism was simply not detected.

The other case involved a 14-year-old TS patient with findings of bilateral gonadoblastoma and a mixed dysgerminoma and embryonal carcinoma [Zelaya et al., 2015]. The patient had a Y chromosome mosaicism, and interphase FISH analysis showed Y chromosomal material in the gonadal tissue; thus, the development of the tumor could be explained by the presence of Y chromosomal material.

In the present case we failed to locate any Y chromosomal material despite an extensive search. The lack of Y chromosomal material questions the current paradigm of the presence of Y chromosomal material being obligatory for the malignant transformation of the gonad in TS. The subsequent analysis of exomes from blood and the tumor revealed an activating mutation in *KIT* in the tumor, which leads to a constitutive activation of the tyrosine kinase. The KIT N655K mutation has been found in GIST patients and melanoma [Minarik et al., 2013]. SCF, which is a KIT ligand, upon binding to KIT results in receptor homodimerization and kinase activation. Mutations result in ligand-independent permanent activation of the KIT tyrosine kinase and are often seen in GIST tumors [Corless et al., 2011]. Importantly, it has been shown that Imatinib, a tyrosine kinase inhibitor, has potent effects on the progression of GISTs and results in dramatic prolongation of the median survival [ESMO/European Sarcoma Network Working Group, 2014] and also in a case of extragonadal seminoma [Pedersini et al., 2007]. It remains to be shown whether the present patient would benefit from Imatinib or other tyrosine kinase inhibitors should she suffer a relapse. CNV analysis showed the presence of several CNV aberrations in the tumor. The most likely pathogenic alteration was probably the fact that 5 copies of *MALAT1* were present, previously linked to an unfavorable survival in epithelial ovarian cancer [Zou et al., 2016].

When studying the literature regarding the presence of Y chromosomal material in patients with TS, the academic debate revolves around the following main subjects. What is the true prevalence of Y chromosomal material in the TS population? What is the actual risk of developing a tumoral gonadal lesion when Y chromosomal material is demonstrated? Should a systematic search for Y chromosome mosaicism be deployed in all patients with TS in whom Y chromosomal material is not demonstrated in their karyotype? The trend in the current debate tends to argue that a systematic search for Y chromosomal material with, for instance PCR and FISH technologies, is justified on the basis that the prevalence of hidden Y chromosome mosaicism is relatively high in the TS population and that the risk of developing a tumoral gonadal lesion in this setting is substantial, although epidemiological data do not suggest an increased risk of ovarian malignancy in TS without Y chromosomal material [Schoemaker et al., 2008]. Current consensus and recent new international guidelines recommend gonadectomy, a low-risk procedure, when Y chromosomal material is demonstrated but do not recommend routine screening for cryptic Y chromosomal material [Gravholt et al., 2017]. However, dysgerminomas can also be seen in normal 46,XX females without Y chromosomal material [Hersmus et al., 2012]. Interestingly, those cases have a high prevalence of activating *KIT* mutations, suggesting an independent mechanism. The presence of a *KIT* mutation supports the idea that the embryonal carcinoma in this patient originates from a primordial germ cell/gonocyte [Hersmus et al., 2016].

Interestingly, besides indeed an activating *KIT* mutation, we also detected an inactivating *AKT1* mutation, which was likely deleterious and not present in a normal population of >140,000 (gnomAD and ExAC) [Lek et al., 2016]. However, in the present setting, this mutation might actually explain the apparent sensitivity to cisplatin, which was part of the first-line treatment, since an activating mutation in *AKT1* has been shown to induce cisplatin resistance [Feldman et al., 2014] although it has to be stated that almost all GCTs (with the exception of teratoma) are sensitive to cisplatin. The variants discovered in *ZNF358* have not been described before, and as such the impact remains to be determined. Both *AKT1* and *ZNF358* are involved in the MAPK pathway via the ubiquitin C protein pathway. This combination of mutations in a single pathway is of interest, because it may enhance the malignant potential of the cells affected.

At the time of diagnosis, the patient's disease was classified as belonging to the poor prognosis germ cell tumor group according to consensus guidelines [Krege et al., 2008]. And by the initiation of the second-line chemotherapy, disease was also considered cisplatin resistant. This is related to 5-year survival rates of 5–10% in the usage of a 2-drug treatment strategy, with the combination of gemcitabine and paclitaxel as a well-established choice [Koychev et al., 2011]. Research is ongoing in order to better understand the phenomenon of resistance, as well as assessing new targets and thus new treatments to overcome it, and thereby improving chances of cure in patients harboring resistance [Jacobsen and Honecker, 2015]. Recommendations on surgical debulking are currently suggested to be carried out at high volume centers [Rice et al., 2014].

In conclusion, we present a rare case of embryonal carcinoma in a patient with TS and a 45,X karyotype, without signs of Y chromosomal material and with an activating *KIT* mutation, perhaps conferring susceptibility to tyrosine kinase inhibitors.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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