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Original Article

Deleterious mutations in esophageal carcinoma cuniculatum detected by next generation sequencing

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Abstract: Esophageal carcinoma cuniculatum (ECC) is a rare form of extremely well-differentiated squamous cell carcinoma of esophagus that is often misdiagnosed preoperatively. The molecular changes underlying ECC remain unknown. This study aimed to explore the molecular signature of ECC using next-generation sequencing (NGS). Five cases of ECC were collected from our pathology database from 2014 to 2019. One patient received chemoradiation and the remaining four patients were treatment-naïve. Areas of normal squamous mucosa, non-invasive component, and invasive component of ECC were circled and macrodissected. Genomic DNA extracted from the macrodissected tissue was sequenced using GatorSeq NGS Panel. Deleterious mutations, predicted by Sorting Intolerant from Tolerant (SIFT), were identified using tumor/normal pairs and annotated by amino acid change. The normal-appearing squamous mucosa in the ECC harbored recurrent deleterious somatic mutations in *ROS1* and *POLE* genes. ECC tumor-specific deleterious mutations were identified on *TP53*, *NOTCH1*, and *PIK3CA* genes. Our results support a mutually exclusive pattern in *NOTCH1* and *PIK3CA* mutation. Non-invasive and invasive components in ECC had identical mutation profiles. Chemoradiation therapy led to disappearance of *NOTCH1* mutation in one ECC case. Our results suggest molecular testing may help pre-operative diagnosis, and provide therapeutic targets in patients with advanced or unresectable ECC.

Keywords: Esophageal carcinoma cuniculatum, esophageal squamous cell carcinoma, next generation sequencing, deleterious mutation, NOTCH1, PIK3CA

Introduction

Esophageal squamous cell carcinoma (ESC) is the most prevalent esophageal cancer worldwide and represents the sixth leading cause of cancer-associated death [1]. It is more commonly found in the upper and middle third of the esophagus, and well-known risk factors include alcohol abuse and tobacco consumption. As a rare variant, esophageal carcinoma cuniculatum (ECC) is a type of extremely well-differentiated ESC [2-6]. Historically, ECC has been considered as a variant of verrucous carcinoma due to similar morphologic and clinical presentation. Fewer than 20 ECC cases have been reported in the literature. As a result, we have limited understanding of its biologic behavior, and early histopathologic diagnosis on mucosal biopsy has proven quite challenging. The majority of the ECC cases have been diagnosed on resection specimen including esophagectomy, endoscopic mucosal resection (EMR), and endoscopic submucosal dissection (ESD) [3].

In an effort to provide semiquantitative method facilitating early histopathologic diagnosis, Chen et al. analyzed a total of 35 esophageal mucosal biopsies for 11 patients with resection-proven diagnosis of ECC [3]. The common histopathologic features of ECC included hyperkeratosis, acanthosis, dyskeratosis, deep keratinization, intraepithelial neutrophils with microabscess, mild cytologic atypia, keratin cyst/furrows, koilocyte-like cells, and lack of atypical mitosis [3]. Applying this semiquantitative histopathologic scheme to esophageal mucosal biopsy has been validated in two ECC cases in a recent report [6].

Progresses in next generation sequencing (NGS) have dramatically advanced our under-

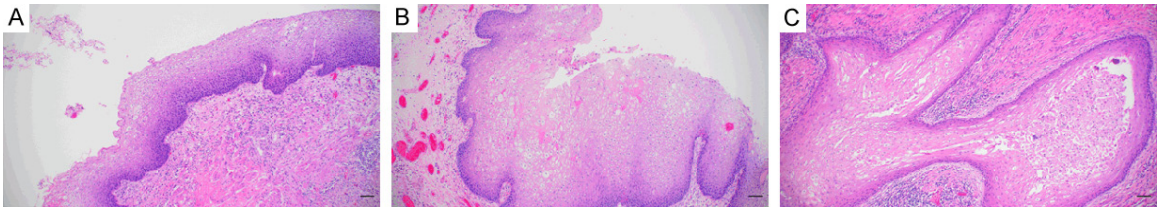

Figure 1. Histology of normal esophageal squamous epithelium (A: H&E stain 100X), non-invasive esophageal carcinoma cuniculatum (B: H&E stain 100X), and invasive esophageal carcinoma cuniculatum (C: H&E stain 100X) used for next generation sequencing. Scale bar: 100 μm.

standing of human cancers through identification of pivotal driver cancer genes and linearization of oncogenic pathways [7-9]. The combination of molecular signature and traditional histomorphologic classification is likely to provide a novel therapeutic approach towards personalized medicine and better clinical outcome. Multiple recurrent genetic alterations have been reported in ESC [10-18], but it was not clear whether cases of ECC were included in those studies. This study aimed to examine the molecular changes in a cohort of ECC tumor samples using NGS.

Materials and methods

Patients and sample collection

A retrospective study was conducted at the University of Florida College of Medicine. Five cases of ECC were collected from our pathology database from 2014 to 2019. They were diagnosed based on the criteria previously published [2, 3]. Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissues. Areas of normal-appearing squamous mucosa (near the ECC site), non-invasive component (above the basement membrane), and invasive component (tumor invading into submucosa) of ECC (**Figure 1**) were circled and macrodissected manually. The research protocol (IRB201901886) was approved by the institutional review boards of the University of Florida (Supplemental Material).

Next generation sequencing

Genomic DNA extracted was sequenced using the GatorSeq NGS Panel covering 177 key cancer genes. Tumor samples were macrodissected and sequenced on the Illumina NextSeq 500 platform following the manufacturer's recommendations to achieve high uniform depth with more than 99% of exons at coverage greater than 100 times. The complete list of cancer genes tested can be found at http://pathlabs.ufl.edu/tests/test-directory-g/gatorseq-ngs/.

Data analysis

NGS data were processed using a customized analysis pipeline (GatorSeq) designed to accurately detect base substitutions and insertions/deletions using human genome version hg19 as the reference. Annotated reports were generated with a GenomeOncology-developed software and database. Deleterious mutations, as predicted by the Sorting Intolerant from Tolerant (SIFT), were identified using tumor/normal pairs and annotated by amino acid change.

Results

Patient characteristics

A total of 5 patients were included in the study, including four females and one male, with the mean age of 60 years (range, 37-73 years; standard deviation 14 years). One patient received chemoradiation and the remaining four patients were treatment naïve. The specimens in this study included esophagectomy specimens (2 cases), an endoscopic mucosal resection (EMR) specimen (1 case), and mucosal biopsy specimens (2 cases) (**Table 1**).

Deleterious mutations in background normal-appearing squamous mucosa

The background normal-appearing esophageal squamous mucosa adjacent to the tumor sites were sequenced as part of the tumor-normal pairs in our ECC cohort. Notably, recurrent genetic alterations were present in these "normal" samples. In normal-appearing squamous mucosa adjacent to the tumor, mutations in

Table 1. Deleterious mutations in ECC

Case	Age	Gender	Specimen Type	Pathology stage	Deleterious mutations (Normal)	Deleterious mutations (Tumor with Normal Subtraction)
1	58	Female	Esophagectomy	ypT3N0	NOTCH3 c.5854G>A; p.Val1952Met NOTCH4 c.4037G>C; p.Arg1346Pro ROS1 c.500G>A; p.Arg167Gln ROS1 c.6637G>A; p.Asp2213Asn FLT1 c.1183G>A; p.Val395Ile	TP53 c.733G>A; p.Gly245Ser (LOF) PIK3CA c.1624G>A; p.Glu542Lys (GOF) MSH6 c.2474T>A; p.Ile825Asn (US)
2	73	Female	Biopsy	N/A	POLE c.4187A>G; p.Asn1396Ser	HRAS c.34G>T; p.Gly12Cys (GOF)
3	67	Female	EMR	N/A	ROS1 c.6637G>A; p.Asp2213Asn ROS1 c.5668A>G; p.Lys1890Glu ROS1 c.433A>C; p.Thr145Pro POLE c.4187A>G; p.Asn1396Ser	TP53 c.817C>T; p.Arg273Cys (LOF) NOTCH1 c.1363G>A; p.Glu455Lys (LOF) KRAS c.35G>A; p.Gly12Asp (GOF) MLH1 c.1930G>A; p.Asp644Asn (US) SETD2 c.6365G>T; p.Arg2122Leu (LOF) SETD2 c.6364C>T; p.Arg2122Trp (LOF)
4	37	Female	Esophagectomy	pT3N0	SDHA c.1337T>C; p.Val446Ala SDHA c.1346C>T; p.Ala449Val SDHA c.1367C>T; p.Ser456Leu SDHA c.1396G>A; p.Ala466Thr	NOTCH1 c.1393 G>A, p.Ala465Thr (LOF)
5	67	Male	Biopsy	N/A	ROS1 c.6637G>A; p.Asp2213Asn TLR2 c.1763_1764delCAinsAG; p.Thr588Lys CSF1R c.1085A>G; p.His362Arg	NOTCH1 c.1253T>C; p.Leu418Pro (US) TLR2 c.1624C>A; p.Leu542Ile (US)

GOF: gain of function; LOF: loss of function; US: uncertain significance.

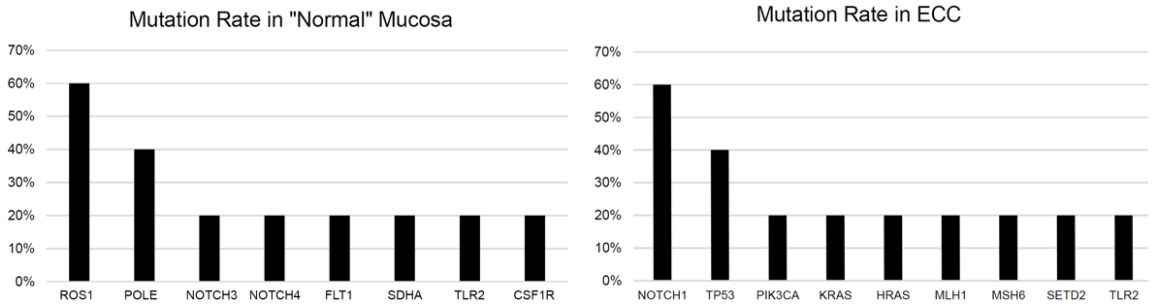

Figure 2. Mutation profiling of ECC and its adjacent "normal" appearing squamous mucosa. Bar plots showing the distribution of mutated genes in 5 ECC samples.

ROS1 (3 cases, 60%) and *POLE* (2 cases, 40%) genes represented recurrent somatic mutations. Other coexisting somatic mutations were found in *NOTCH3*, *NOTCH4*, *FLT1*, *SDHA*, *TLR2*, and *CSF1R* gene (**Table 1**; **Figure 2**).

Deleterious mutations in ECC tumor samples

To identify tumor specific somatic mutations, the DNA sequences from tumor samples were compared with their paired normal samples followed by subtraction of the common variants shared by both samples. As shown in **Table 1** and **Figure 2**, the most common deleterious gene mutations in ECC were missense mutations in *NOTCH1* gene (3 cases, 60%) and Tumor protein 53 gene (*TP53*) (2 cases, 40%). Other deleterious mutations were found in phosphoinositide-3-kinase catalytic alpha polypeptide (*PIK3CA*), *KRAS*, *HRAS*, *SETD2* and *TLR2* gene.

Deleterious mutations in non-invasive and invasive components of ECC

Two ECC cases in the ECC cohort had both non-invasive and invasive components. Compared with normal esophageal squamous mucosa (**Figure 1A**), both non-invasive component (**Figure 1B**) and invasive component (**Figure 1C**) of ECC shared similar histologic features including acanthosis, dyskeratosis, mild cytologic atypia, with koilocyte-like cells. Not uncommonly, the diagnosis of frank invasion in ECC cases could be made only with a resection specimen. In our study, the background normal squamous mucosa, non-invasive in situ component of ECC, and invasive component of ECC were con-

Table 2. Comparison of mutation profile between non-invasive and invasive components in ECC

Case	Non-invasive component	Invasive component
1	TP53 c.733G>A; p.Gly245Ser (LOF) PIK3CA c.1624G>A; p.Glu542Lys (GOF) MSH6 c.2474T>A; p.Ile825Asn (US)	TP53 c.733G>A; p.Gly245Ser (LOF) PIK3CA c.1624G>A; p.Glu542Lys (GOF) MSH6 c.2474T>A; p.Ile825Asn (US)
3	TP53 c.817C>T; p.Arg273Cys (LOF) NOTCH1 c.1363G>A; p.Glu455Lys (LOF) KRAS c.35G>A; p.Gly12Asp (GOF) MLH1 c.1930G>A; p.Asp644Asn (US) SETD2 c.6365G>T; p.Arg2122Leu (LOF) SETD2 c.6364C>T; p.Arg2122Trp (LOF)	TP53 c.817C>T; p.Arg273Cys (LOF) NOTCH1 c.1363G>A; p.Glu455Lys (LOF) KRAS c.35G>A; p.Gly12Asp (GOF) MLH1 c.1930G>A; p.Asp644Asn (US) SETD2 c.6365G>T; p.Arg2122Leu (LOF) SETD2 c.6364C>T; p.Arg2122Trp (LOF)

GOF: gain of function; LOF: loss of function; US: uncertain significance.

Table 3. Comparison of mutation profile between pre- and post-chemoradiation therapy in ECC

Case 5	Deleterious mutations (pre-chemoradiation therapy)	Deleterious mutations (post-chemoradiation therapy)
Normal	ROS1 c.6637G>A; p.Asp2213Asn TLR2 c.1763_1764delCAinsAG; p.Thr588Lys CSF1R c.1085A>G; p.His362Arg	ROS1 c.6637G>A; p.Asp2213Asn TLR2 c.1763_1764delCAinsAG; p.Thr588Lys CSF1R c.1085A>G; p.His362Arg
Tumor	NOTCH1 c.1253T>C; p.Leu418Pro (US) TLR2 c.1624C>A; p.Leu542Ile (US)	N/A

GOF: gain of function; LOF: loss of function; US: uncertain significance. The deleterious mutations in tumor samples represent subtraction from the corresponding normal tissue.

comitantly sequenced. For both ECC cases, the non-invasive and invasive components demonstrated identical mutation profiles with one case showing deleterious mutation in *MSH6*, *PIK3CA*, and *TP53* gene, and the other case in *NOTCH1*, *SETD2*, *KRAS*, *MLH1*, and *TP53* genes (**Table 2**).

Deleterious mutations in ECC after chemoradiation therapy

One patient in our study had pre-operative biopsy (treatment naïve), underwent chemoradiation therapy, and then had surgery. Comparing the NGS results revealed almost identical deleterious gene mutation profiles between pre- and post-chemoradiation therapy tumor samples, with the only exception being that the deleterious mutation of the *NOTCH1* gene disappeared upon chemoradiation therapy (**Table 3**).

Discussion

Carcinoma cuniculatum was first described in 1954 as a variant of squamous cell carcinoma peculiar to the foot [19]. Subsequently, carcinoma cuniculatum has been reported in a variety of non-cutaneous anatomic sites including head and neck, esophagus, cervix, among others [2-6, 20-22]. Carcinoma cuniculatum of the esophagus is a type of extremely well-differentiated squamous cell carcinoma with low malignant potential. None of the reported ECC cases had lymph node metastasis [2, 3, 6]. The surgical resection of ECC provided excellent long-term survival even in patients with advanced disease [2-6]. Its indolent clinical behaviors sharply contrast with the aggressive nature and poor survival observed in conventional ESC. Given the rarity of ECC, there is the lack of standard therapy. Two cases have been reported to undergo neoadjuvant chemoradiation therapy and subsequently esophagectomy, and both cases had residual adventitia-invading tumor on the esophagectomy specimens [6]. Therefore, ECC may be less responsive to neoadjuvant chemoradiation therapy as compared to conventional esophageal squamous cell carcinoma. ECC remains a histopathologic challenge for surgical pathologists, especially when dealing with superficial mucosal biopsy specimens, due to its low incidence and deceptively bland histomorphologic features.

To identify gene mutations associated with tumor initiation, progression, and prognosis of ESC, several large-scale genomic studies have been conducted. Multiple recurrent gene mutations have been identified in ESC, with muta-

tions in *TP53*, *NOTCH1*, *PIK3CA*, *FAT1*, *CDKN2A*, and *FBXW7* genes being the most common [10-18]. However, it is not clear whether those studies included ECC as no such data were given. In this study, we conducted the first NGS analysis to explore the molecular signature in ECC. Molecular comparison between our ECC mutational profile and the published ESC data reveals significant overlap in recurrent genetic alterations. Similar to the conventional ESC, ECC frequently harbors mutations in *TP53* (40% of cases in the current study), *NOTCH1* (60% of cases in the current study) and *PIK3CA* gene (20% of cases in the current study).

As the guardian of the genome, *TP53* plays an important role in regulating cell cycle arrest, programed cell death, and cellular senescence. *TP53* is the most commonly mutated gene in ESC (60% to 92%) in multiple large-scaled studies [8]. Loss of function mutations in *TP53* gene occur early in ESC carcinogenesis and have been associated with its poor prognosis [23-26]. *PIK3CA* encodes the 110 kDa catalytic subunit of the phosphatidylinositol 3-kinase (PI3K), and is also one of the most mutated genes in ESC (5% to 17%) [8]. The PI3K pathway plays a pivotal role in tumorigenesis of human cancer by regulating multiple cellular processes including metabolism, cell cycle, motility, and survival [27].

Unlike *TP53* and *PIK3CA* gene that are also frequently mutated in esophageal adenocarcinoma (EAC), mutations in *NOTCH1* gene are not present in EAC, and therefore represent a more specific molecular event in ESC [10, 12]. *NOTCH1* is the second most commonly mutated gene in ESC (8% to 32%) [8], and is associated with tumor grade, stage, and poor survival [28]. Possible underlying mechanisms include its roles in regulating stem cell homeostasis, epithelial-to-mesenchymal transition, and resistance to chemoradiation therapy [29]. A high rate (60%) of *NOTCH1* mutation is present in ECC in our study. All of the *NOTCH1* mutations in our study (*NOTCH1*:c.1363G>A, p.Glu455Lys; *NOTCH1*:c.1393G>A, pAla465Thr; and NOTCH1:c.1253T>C, p.Leu418Pro) are missense mutations involving the epidermal growth factor (EGF)-like ligand-binding domain and thus of potential detrimental consequence in cell growth leading to tumor formation. Of note, two of the *NOTCH1* mutations (*NOTCH1*:c.1363G>A, p.Glu455Lys and *NOTCH1*: c.1393G>A, pAla465Thr) have been reported in conventional ESC samples (13). The NOTCH1 mutation (c.1253T>C, p.Leu418Pro) appears unique to ECC and has never been retrieved in ESC and other tumor samples on the Cancer Genome Atlas (TCGA) database.

Our study demonstrates the enrichment of deleterious mutations in *NOTCH1* and *PIK3CA* gene in ECC. When categorizing tumor through mutation status of *NOTCH1* and *PIK3CA* gene, the majority of ESC cases (62% to 86%) in previous studies have wild type expression of *NOTCH1* and *PIK3CA* gene [8, 10, 15-18, 30]. However, only one case in our ECC cohort (20%) had wild type expression of *NOTCH1* and *PIK3CA* gene (Case#2, **Table 1**). In our study, none of the cases with mutations in *NOTCH1* gene showed concomitant mutations in *PIK3CA* gene. Our results confirm a prior study suggesting mutually exclusive mutational pattern of *NOTCH1* and *PIK3CA* gene in ESC [30]. Of note, a significant difference in clinical outcome was proposed among patients with mutations in the *NOTCH1* or *PIK3CA* gene. Patients harboring mutations in *NOTCH1* gene more likely have well differentiated tumor, early tumor stage and are negative for lymph node metastasis, but have a poor response to chemotherapy and poor clinical outcome. On the other hand, patients harboring mutations in *PIK3CA* gene more frequently have good response to chemotherapy, and *PIK3CA* mutations could serve as a favorable prognostic biomarker in some studies [30, 31]. The difference in molecular signature, especially the *NOTCH1/PI3KCA* mutation profile, may account for the unique histomorphology and clinical outcome in ECC.

Our study also demonstrates an identical mutation profile between non-invasive and invasive components within ECC. ECC is an extremely well-differentiated squamous cell carcinoma. Therefore, to make a definitive pathologic diagnosis of ECC and to document its invasive nature on a mucosal biopsy specimen could be challenging. Our results suggest that it is practical to perform molecular testing for the preoperative diagnosis even with superficial mucosal biopsy specimen in patients with progressive dysphagia who already had multiple "non-diagnostic" biopsies due to deceptively bland histomorphology of squamous epithelium. Our study also reveals the dynamic change in *NOTCH1* mutations after chemoradiation thera-

py. The ECC cohort in this study included a 67-year-old male with pre-operative mucosal biopsy followed by chemoradiation therapy and surgical resection. The mutation profile between pre- and post-operation is quite identical, with the only exception of the disappearance of *NOTCH1* and *TLR2* mutation after chemoradiation therapy. Given the frequent NOTCH1 mutation observed in ECC, NOTCH1 mutation was assumed to be the "driver mutation" and the TLR2 to be the "passenger mutation" in this specific case. Notch pathway is associated with chemoresistance and tumor survival through maintaining cancer stem cells [29]. The loss of *NOTCH1* mutations after therapy has been reported in chronic lymphocytic leukemia patients [32, 33], presumably reflecting the complex fluctuation during tumor clonal evolution. The disappearance of *NOTCH1* mutation presumably would lead to an increased sensitivity towards chemoradiation therapy, but the exact clinical impact of such change awaits further investigation.

Our study also, for the first time, revealed the molecular signature of "normal" squamous mucosa among ECC cases. All "normal" squamous mucosa analyzed by NGS were taken near the ECC tumor site. This "quiet but not quiescent" phenomenon has been proposed previously. For example, Martincorena et al. demonstrated that a high burden of mutations was already present in normal human skin tissue before cancer development. The most significant mutations in their study, including *NOTCH1*, *NOTCH2*, *NOTCH3*, *TP53* and *FAT1* mutations, were known to drive carcinogenesis of cutaneous squamous cell carcinoma [34]. In our ECC cohort, normal-appearing esophageal squamous mucosa also harbored multiple recurrent somatic mutations (such as *ROS1* and *POLE*). *ROS1* is a receptor tyrosine kinase which mutations and gene rearrangements have been reported in cancers such as non-small cell lung carcinoma, colorectal carcinoma, melanoma, and inflammatory myofibroblastic tumours. *ROS1* positive tumors are amenable to targeted therapy by crizotinib [35]. On the other hand, mutations of *POLE*, the gene encoding the DNA polymerase ε that is involved in DNA replication and repair, have caused accumulation of DNA errors that lead to ultra-mutated tumors. Somatic mutations of POLE are more commonly seen in colorectal and endometrial cancers [36]. Interestingly, mutations in *ROS1* and *POLE* genes have not been reported in conventional ESC [8, 10, 12-18]. Whether *ROS1* and *POLE* mutations in the adjacent normal esophageal squamous mucosa laid the foundation of ECC pathogenesis still awaits further investigation. Somatic mutations in multiple NOTCH pathway genes including *NOTCH3* and *NOTCH4* were also present. Interestingly, none of the normal samples carried *NOTCH1* mutation, supporting its role in driving ECC carcinogenesis. Accumulation and combination of these somatic mutations within normal tissue may be the key to understanding the mutant clonal expansion and tumorigenesis in ECC.

Our study has several strengths. First, it is the first molecular study specifically performed on ECC. All ECC cases were diagnosed and re-reviewed by a gastrointestinal pathologist with expertise in this entity. All tissue areas used for NGS were circled. The limitations of our study include small size of the cohort and lack of normal esophageal mucosa from subjects without ECC to delineate the significance of "normal" esophageal squamous mucosa from ECC patients in our study.

In summary, ECC harbors hot spot somatic mutations commonly found in conventional ESC, including *TP53*, *NOTCH1* and *PIK3CA*. Unlike conventional ESC in which the majority of cases (62% to 86%) have wild type expression of *NOTCH1* and *PIK3CA* gene [8, 10, 15-18, 30], the majority of the ECC cases (80%) in our study harbored either a *NOTCH1* or *PIK3CA* mutation. Mutations in *NOTCH1* and *PIK3CA* in ECC appear mutual exclusive to our study. A unique molecular signature is also found in the normal-appearing esophageal squamous mucosa among our ECC cohort. This study will not only provide proof-of-concept for the preoperative molecular diagnosis of ECC, but also extends our knowledge of ECC carcinogenesis and tumor clonal evolution. This may lead to therapeutic targets in patients with advanced or unresectable ECC.

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Disclosure of conflict of interest

None.

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