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RESEARCH PAPER

Absence of germline *CDKN2A* mutation in Sicilian patients with familial malignant melanoma: Could it be a population-specific genetic signature?

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ABSTRACT

Germline *CDKN2A* mutations have been described in 25% to 40% of melanoma families from several countries. Sicilian population is genetically different from the people of Europe and Northern Italy because of its historical background, therefore familial melanoma could be due to genes different from high-penetrance *CDKN2A* gene. Four hundred patients with cutaneous melanoma were observed in a 6-years period at the Plastic Surgery Unit of the University of Palermo. Forty-eight patients have met the criteria of the Italian Society of Human Genetics (SIGU) for the diagnosis of familial melanoma and were screened for *CDKN2A* and *CDK4* mutations. Mutation testing revealed that none of the families carried mutations in *CDK4* and only one patient harboured the rare *CDKN2A* p.R87W mutation. Unlike other studies, we have not found high mutation rate of *CDKN2A* in patients affected by familial melanoma or multiple melanoma. This difference could be attributed to different factors, including the genetic heterogeneity of the Sicilian population. It is likely that, as in the Australian people, the inheritance of familial melanoma in this island of the Mediterranean Sea is due to intermediate/low-penetrance susceptibility genes, which, together with environmental factors (as latitude and sun exposure), could determine the occurrence of melanoma.

Introduction

Melanoma is a high-grade, poorly differentiated malignant tumor of melanin pigment-producing cells (melanocytes) with poor prognosis in the metastatic stage, accounting for more than 70% of the skin cancer related deaths.[1] A familial history of melanoma is a strong predictor of melanoma development. Up to 10% of all cases of cutaneous malignant melanoma occurs in a familial setting. Familial genetic linkage studies have led to the identification of 2 high-penetrance susceptibility genes, *CDKN2A* and *CDK4*, involved in senescence and cell cycle arrest. Rare mutations or deletions in these genes confer an elevated risk of developing melanoma.[2,3]

CDKN2A (cyclin-dependent kinase inhibitor 2A) is known to be a major high-risk melanoma susceptibility gene that it is transmitted according to a dominant mode if inherited in melanoma-prone families. The *CDKN2A* gene is located on chromosome 9p21 and includes 4 exons (1α, 1β, 2 and 3) encoding for 2 tumor-suppressor proteins, INK4A (p16^{INK4a}) and ARF (p14^{ARF} in humans and p19^{ARF} in mice), which are translated from alternative spliced transcripts.[4,5] Most germline mutations that confer melanoma risk occur in exons 1α and 2, indicating that p16INK4a is the preferentially targeted and functionally dominant component of *CDKN2A*.[6,7] Rare deletions at the exon 1beta have also been reported, suggesting that p14ARF

is a melanoma susceptibility gene independent from p16INK4a.[8-10] Even rarer deep intronic mutations of *CDKN2A* have also been described, although these account for very few cases worldwide.[11-14]

INK4A is a cyclin-dependent kinase inhibitor that activates the pRB protein via negative regulation of Cdk4/6, promoting the progression through the G1/S transition, whereas p14ARF interacts with the MDM2 protein, whose principal function is to promote the ubiquitin-mediated degradation of the tumor-suppressor protein p53.[15,16] Thus, the loss of function of p16^{INK4a} promotes CDK4 and CDK6 activation, causing hyper-phosphorylation and inactivation of retinoblastoma protein (pRB), and activation of E2F1 (**Fig. 1**). Indeed, several genetic and epigenetic analyses showed that *INK4A* is lost in 50% of melanoma cases, inactivated by methylation of its promoter in about 10% of tumors or inactivated by point mutations in approximately 9% of melanoma cases.[17] The frequent and specific inactivation of p14ARF in melanoma supports its considerable role in melanomagenesis.[18-20] Additionally, BRAF-activating mutations and functional loss of p16^{INK4a} and p14^{ARF} were found in the majority of melanomas.[21] Binni et al.[22] have reported that overall frequency of p14ARF mutations in Italian familial melanoma is 3.2%.

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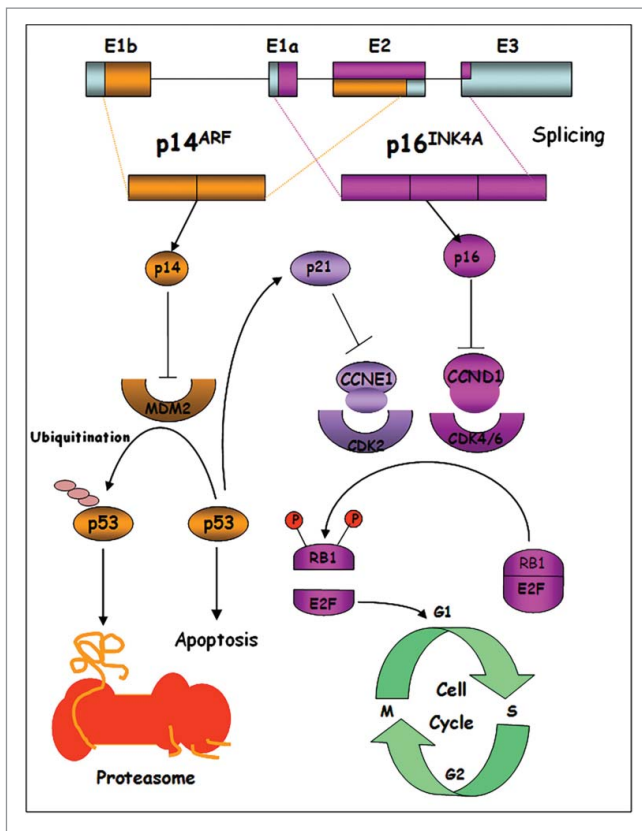*Sara Di Lorenzo and Daniele Fanale contributed equally to this work.

Figure 1. *CDKN2A* locus structure and signaling pathways regulated by p16INK4a and p14ARF.

Germline mutations in high-susceptibility *CDK4* gene are rare, as reported in literature, and inhibit the regulation of the protein by p16^{INK4a}, but maintain the interaction between CDK4 and cyclin D1, leading to constitutive activation of the complex and aberrant proliferation, through pRB inactivation and activation of the transcription factor E2F. E2F activates the transcription of S-phase genes, thereby promoting cell proliferation.[23] Moreover, Rane et al. reported that *CDK4* mutations promote tumorigenesis and determine escape from the cellular senescence mechanism of melanocytes transplanted into nude mice.[24]

During the last years, genome-wide association studies (GWAS) have allowed to identify common single nucleotide polymorphisms (SNPs) associated with a low risk of developing cancers, including melanoma. Therefore, the identification of genetic variants with low/intermediate allele frequency conferring a moderate risk of cancer represents an important scientific approach to discover novel melanoma-predisposing genes.[25,26] Among these, 2 low/intermediate-penetrance susceptibility genes, *MITF* (microphthalmia-associated transcription factor) and *MC1R* (melanocortin 1 receptor), play an important role in the development of melanoma. Genomic amplification of *MITF* was detected in 10% of primary tumors and 20% of metastatic melanomas and correlates with decreased 5-year overall patient survival.[27,28] MITF belongs to the MYC supergene family of basic helix-loop-helix transcription factors and is involved in proliferation control and survival.[29] A model, in which an increase in MITF expression is associated with the differentiation,[30] while moderate MITF

levels are linked with proliferation,[31] and transient low MITF levels with a melanoma-initiating cell phenotype has been suggested.[32] MITF activity is regulated by posttranslational modifications such as phosphorylation and degradation via the ubiquitin-proteasome pathway in response to activation of the ERK pathway.[33] In patients with a strong family history a germline missense substitution p.E318K in *MITF*, conferring a fold5- increased melanoma risk, was identified.[34,35] This mutation confers invasive and migrative properties to melanoma cells, providing tumor progression abilities. MITF can control the expression of several genes involved in cell survival (*HIF-1α*, *BCL⁻2*, *MET*, *APE-1*),[36-38] cytoskeleton remodeling and migration,[39] and cell proliferation (*CDK2*).[40] Moreover, MITF activity is correlated with the resistance to UV-induced apoptosis in melanocytes.

MC1R is a transmembrane G protein-coupled receptor expressed on the cell surface of epidermal melanocytes, which, upon hormonal stimulation, actives the adenylate cyclase and the cAMP/PKA/CREB pathway.[41] Allelic variants of *MC1R* are an important risk factor for melanoma, and the melanoma penetrance increases in presence of a *CDKN2A* mutation.[42] Other low-risk allelic variants are responsible for differences in pigmentation of skin, hair, and eyes, and determine variations in skin sensitivity to UV, by increasing the melanoma risk.

Sun exposure is widely considered as the critical environmental risk factor for cutaneous malignant melanoma, which originates as a consequence of deleterious interactions between ultraviolet (UV) radiations and the melanocyte genome. In fact, UV radiations may contribute to melanoma development through combined genotoxic and mitogenic effects in melanocytes.[43] The combination of inherited intermediate/low-penetrance allelic variants with environmental factors such as sun exposure might induce the onset of melanoma.[44]

In this work we performed a screening of germline *CDKN2A* and *CDK4* mutations in 48 Sicilian patients with familial malignant melanoma in order to assess the frequency and influence of these high-risk susceptibility genes in individuals belonging to this specific geographical area.

Results

Clinicopathological features of the familial melanomas in sicilian population

Forty-eight Sicilian patients who have met the selection criteria for familial melanoma genetic testing have been studied, by defining the clinical and pathological features of their melanomas. Of these patients, twenty-four were men and twenty-four were women, with a mean age at the onset of 35,8 y (range 15-60). The site of primary melanoma was the trunk in 56% of patients (27), limbs in 40% (19) and head and neck in 4% (2). In the patients enrolled in the study the most common form of melanoma was superficial spreading melanoma (52%), followed by nodular melanoma (27%), and *in situ* melanoma (21%). No cases of lentigo maligna melanoma (LMM) and acral lentiginous melanoma (ALM) were observed among patients who met the selection criteria for genetic counseling for familial melanoma. In only 3 cases 2 primary melanomas were diagnosed at the

same time (synchronous primary melanomas). In 7 cases the second melanoma appeared in a different anatomical area than the area of primary lesion, within 5 y from diagnosis of the first melanoma. The tumor thickness measurement according to Breslow thickness highlighted that about 60% of patients presented a thin melanoma at the diagnosis with a better prognosis than other patients with a thickest melanoma. The AJCC Melanoma staging showed that almost all patients (44) had a localized melanoma at the diagnosis (stages 0-II), while only 4 patients had an advanced-stage melanoma (stages III and IV). However, the fact that most of patients showed an early-stage melanoma at diagnosis is likely due to a greater attention of individuals with family history in controlling (by means of periodic cutaneous screening) and removing suspected skin lesions.

Furthermore, in 70% of patients, in addition to melanoma, one or more dysplastic nevi have been removed (**Table 1**).

Table 1. Clinical and pathological features of familial melanoma patients

	No. of patients
All patients	48
Sex	
Male	24
Female	24
Site of primary MM	
Head and neck	2
Trunk	27
Limbs	19
Histologic subtype	
SSM	25
NM	13
In situ	10
Number of primary MM	
Single	38
Multiple	10
- Synchronous	3
- Metachronous	7
Breslow thickness	
In situ	10
< 1 mm	19
1-2 mm	9
> 2 mm	10
AJCC Stage #	
0	10
Ia	14
Ib	13
IIa	3
IIb	4
III	3
IV	1
DN	
Yes	34
No	14
Age at diagnosis (years)	
< 20	5
20–30	7
30–40	19
40–50	10
> 50	7
Other cancers	3
Chronic Myeloid Leukemia	1
Breast Cancer	2

Disease stage was defined according to the recent American Joint Committee on Cancer (AJCC) guidelines.
Abbreviations: MM, malignant melanoma; SSM, superficial spreading melanoma; NM, nodular melanoma; DN, presence of dysplastic nevi.

Screening of germline CDKN2A and CDK4 mutations

A total of 48 familial malignant cutaneous melanoma cases from Sicilian patients was screened for germline mutations in *CDKN2A* exons 1α, 1β, 2 and 3 and *CDK4* exon 2 in order to evaluate the frequency of these high susceptibility genes in individuals belonging to this specific geographical area. Mutation analysis revealed that none of the examined families carried mutations in exon 2 of *CDK4* and only one patient harboured a rare missense mutation in exon 2 of *CDKN2A*.[45] The patient was a carrier of the p.R87W mutation (*c.259C > T*), which causes the substitution of an arginine for a tryptophan at position 87, instead of the most common p.G101W mutation (*c.301 G > T*), which causes the substitution of a glycine for a tryptophan at position 101.[46] At the age of 49 y (first diagnosis), the mutation carrier patient had a superficial spreading melanoma (SSM) in the trunk with a Breslow thickness of 1.9 mm. The proband then developed a second metachronous melanoma at the age of 51 y and a third melanoma at the age of 54 y Both melanomas were *in situ* melanomas of the back and eyelid, respectively.

Since mutational screening of relatives of individuals harbouring germline mutations in susceptibility genes could even improve prevention and surveillance strategies of melanoma through early identification of the pigmented skin lesions at risk of malignant transformation, we performed a survey about the relatives of the *CDKN2A* germline mutation carrier patient. After a careful study of all the relatives of the proband, the same gene mutation was detected in his sister and his daughter, unlike other families who showed no germline mutations. This mutation has not been previously described in the literature, except in a few cases of melanoma.[47] Furthermore, proband's sister developed an early-stage melanoma (*in situ*), while his daughter was an unaffected mutation carrier (**Fig. 2**).

Overall, our data indicate that a high mutation rate of *CDKN2A* was not found in Sicilian patients affected by melanoma who met criteria for the diagnosis of familial or multiple melanoma, in contrast to what reported in other studies.[48]

Discussion

Melanoma is the most serious and aggressive form of skin cancer whose incidence has been continuously increasing in the last decades, and faster than any other cancers. For patients with cutaneous melanoma, the prognosis is related to the location and depth of the primary tumor, and the presence or absence of locoregional and distant metastatic disease.[49]

Melanoma is the most dangerous form of skin cancer in the white population, being largely resistant to conventional therapies at advanced stages. The management of patients with advanced melanoma represents a significant challenge considering that, historically, chemotherapy and immunologic therapies have produced only modest results in the treatment of metastatic melanoma.[50,51] Patients with metastatic melanomas have a median survival rate that typically ranges from 6 to 10 months. Currently, prevention and early detection represent the only effective strategies to reduce the incidence of this tumor. Despite improvements in early melanoma diagnosis, the 5-year survival rate remains low in advanced disease.[52,53]
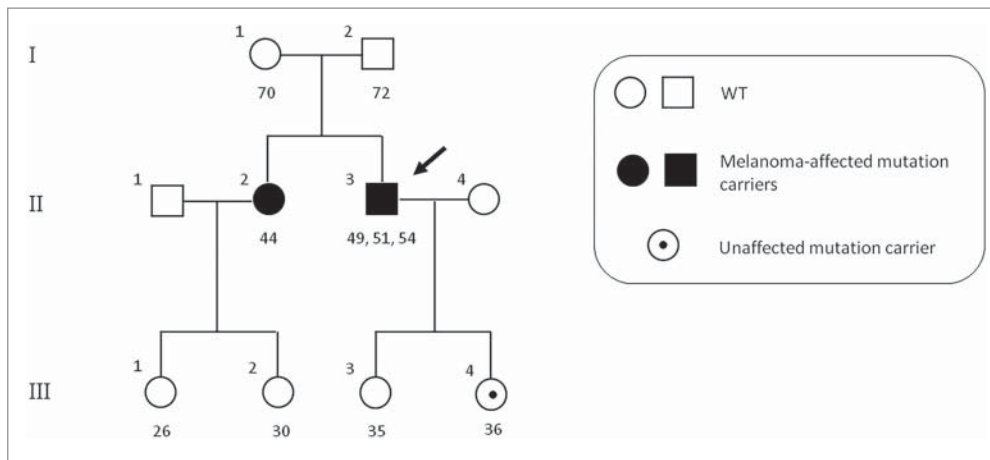
Figure 2. Pedigree of the *CDKN2A* p.R87W mutation-positive family. Index case is indicated by an arrow. Numbers under symbols show the age for the relatives and age at diagnosis for the affected individuals. For patients with multiple melanomas, different ages are separated by commas. WT, non-carriers of mutation.

Germline mutations in genes encoding for CDK4 and CDKN2A, involved in regulation of the cell cycle, have been shown to confer a high risk of malignant melanoma.[54] In addition, frequent germline allelic variants in the *Casp8*, *MTAP*, *MATP*, *MC1R*, *MITF* and *ASIP* genes have been identified as low-risk susceptibility genes or as modifiers of high-risk susceptibility genes.[55]

Germline *CDKN2A* mutations have been described in 25-40% of melanoma-prone families from several countries, and most of them are missense mutations mainly affecting p16INK4a. Typically, in familial melanoma there are more family members affected by melanoma with early-onset (particularly with a vertical pattern of inheritance). High number of cases of melanoma in the same family, early age of onset and presence of multiple primary melanoma (sincronous or metacronous) showed significant association with *CDKN2A* mutations.[56] Approximately from 3% to 5% of all patients with melanoma will develop additional primary melanomas in their lifetime. The prevalence of *CDKN2A* mutations increases with the number of primary melanoma diagnoses in the individuals. For familial cases with *CDKN2A* mutation carriers the overall penetrance has been estimated to be 30% by age 50, and 67% by age 80, although this risk is higher in subjects who live in sunnier climes. Melanoma risk varies by geographic area as showed by studies of families with *CDKN2A* mutations from North America, Europe and Australia. The reasons for these variations are not well understood, however, there may be differences in the sun exposure, other genetic or individual alterations, or a combination of these factors.[57,58] The probability of *CDKN2A* mutation in familial melanoma is higher in areas at low incidence of melanoma as Europe (57%) and North America (45%) than in areas at high incidence as Australia (20%). In these high-incidence geographical areas there may be a combined effect of moderate/low-penetrance susceptibility genes and higher sun exposure (environmental effects on susceptibility).[59]

A high prevalence of the p.G101W germline mutation in the *CDKN2A* (p16INK4a) gene was detected in a large number of melanoma families worldwide from different countries such as Italy, Spain, France, United Stated, Israel,

Australia.[46,47] Haplotype analysis suggested that this mutation is a common founder rather than a mutational hotspot.[60]

We studied 400 melanoma cases enrolled in a 6-years period at the Plastic Surgery Unit of the University of Palermo and selected 48 patients who met the criteria for familial melanoma genetic testing, in order to investigate the *CDKN2A* and *CDK4* genes for germline mutations. The performed analyses showed that almost all patients (except one) with familial cutaneous melanoma were negative for *CDKN2A* mutation screening, while no variants were identified in *CDK4* exon 2 of all patients, suggesting that both genes have a low impact in melanoma susceptibility of Sicilian patients. The only carrier of *CDKN2A* germline mutation harboured a rare missense mutation in exon 2 (p.R87W) instead of the most frequent p.G101W founder described in literature and detected in 60% of familial melanoma cases in Italy [61,62] (**Fig. 3**). This p.R87W rare missense mutation was found with very low frequency also in other countries belonging to Mediterranean geographical area, including Greece (Athens) and Spain (Barcelona).[47,63,64] *In vitro* experiments showed that p.R87W mutation alters both the cell cycle and reactive oxygen species (ROS) protective functions of p16INK4a.[45]
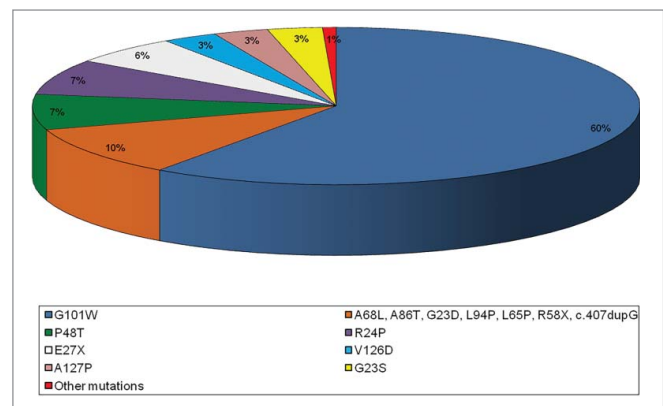

Figure 3. Pie chart distribution of *CDKN2A* missense mutations detected in Italian familial melanomas.

Unlike individuals belonging to other European and Italian regions, the examined Sicilian families exhibited a very low frequency of the *CDKN2A* mutation. This difference could be attributed to different factors such as, for example, the historical background of the Sicily and its crucial geographical position in the center of Mediterranean Sea, cradle of several peoples, cultures and civilizations. In fact, the different dominations (Greeks, Phoenicians, Etruscans, Romans, Byzantines, Arabs, Normans, Aragon, Bourbons) are responsible for the genetic heterogeneity of the Sicilian people, already described for allelic variants of hemoglobin in Beta Thalassemia.[65] In addition, Salemi et al.[66] have reported that Xq27 region, containing the SPANX (sperm protein associated with the nucleus in the X chromosome) gene family, may be involved specifically in melanoma development in Sicilian male population. The latitude and higher sun exposure in our region than in other parts of Italy or Europe, probably represent the environmental factors causing the loss of heterozygosity (LOH) of moderate/low-penetrance susceptibility genes promoting the onset of melanoma. For these reasons, dermatological screening, sun exposure avoidance and self-examination are encouraged even in patients with familial melanoma negative for *CDKN2A* mutation screening.

Furthermore, other Italian regions belonging to Mediterranean Basin including Sardinia (Sassari) and Campania (Naples) showed a great similarity with the Sicily, exhibiting a null or very low frequency, respectively, of the *CDKN2A* germline mutation in melanoma families.[67,68]

Our findings have led us to hypothesize that a very low frequency of germline *CDKN2A* mutations could be a population-specific genetic signature for Sicilian patients affected by familial melanoma.

A larger number of patients enrolled for genetic testing and the introduction of new genetic screenings to identify other mutations in other moderate/low-penetrance susceptibility genes involved in the genesis of familial melanoma will aid in clarifying which are the most common gene mutations and the role of environmental factors in determining this disease.

Patients and methods

Sample collection

In the period from January 2009 to December 2014, 400 patients affected by cutaneous melanoma were observed and treated in the Plastic Surgery Unit at the Department of Surgical, Oncological and Oral Sciences of the University of Palermo. Forty-eight patients have met the following criteria for familial melanoma genetic test in accordance with the recommendations of the Italian Society of Human Genetics (SIGU, www.sigu.net/show/attivita/5/1/linee%20guida?page=5) and Melanoma Genetics Consortium (GenoMEL, www.genomel.org)[69]:

-Two or more individuals affected by melanoma in the same family (first degree relative);

-Multiple primary melanoma in the same patient with an early age of onset;

-Early-onset melanoma patient and pancreatic cancer in a member of the same family;

-Dysplastic nevus syndrome and a relative with a melanoma.

This data is coherent with that reported in the literature, in fact, about 10% of malignant melanoma occurs in familiar setting.

Patients with sporadic melanoma were excluded from the study. Diagnosis of melanoma was confirmed by histologic analysis. All clinical information for each enrolled patient was recorded anonymously and coded. All recruited individuals were from a Southern Italian region (Sicily, Palermo). A written informed consent was obtained from each investigated patient and the study was approved by ethical committee of the university-affiliated hospital. Patients were further informed that a negative screening for *CDKN2A* and *CDK4* can be void of significance, as other unknown susceptibility genes may be involved.

Clinicopathological features of the familial melanoma patients were summarized in **Table 1**.

Mutation screening by sequencing analysis

Germline mutation screening of the *CDKN2A* and *CDK4* genes was carried out. Genomic DNA was extracted from whole peripheral blood of patients with familial melanoma using the QIAamp Blood Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The DNA yields and purity were determined spectrophotometrically by measuring the absorbance of aliquots at 260 and 280 nm. All DNA samples were of sufficient quality to be genotyped. Direct sequencing of the PCR products of 4 exons (1α, 1β, 2, 3) of *CDKN2A* gene and exon 2 of *CDK4* gene was performed using a BigDye Terminator v3.1 and then sequencing by ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Each genetic variant was confirmed by direct sequencing analysis on 2 independent peripheral blood samples. Data analysis was performed using the Sequencing Analysis 5.1.1 and Run 3100 Data Collection v2.0 softwares.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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