GENES, CHROMOSOMES & CANCER 45:791–797 (2006)

RESEARCH ARTICLES

Prevalence of *BRCA1* Genomic Rearrangements in a Large Cohort of Italian Breast and Breast/Ovarian Cancer Families Without Detectable *BRCA1* and *BRCA2* Point Mutations

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The presence of genomic rearrangements of the *BRCA1* gene in breast and/or ovarian cancer families has been intensively investigated in patients from various countries over the last years. A number of different rearrangements have been reported by several studies that clearly document the involvement of this mutation type in genetic predisposition to breast and ovarian cancer. Population-specific studies are now needed to evaluate the prevalence of genomic rearrangements before deciding whether to include *ad hoc* screening procedures into standard diagnostic mutation detection approaches. Indeed, the vast majority of the studies have been performed on small, highly selected, sample sets because of the limitations imposed by the laborious technical approaches. Moreover, prevalence figures are likely to differ across different countries according to the ethnic origin of each specific population. Here we analyze a large cohort of 653 Italian probands, negative for *BRCA1* and *BRCA2* point mutations, gathered from four National Institutions. We report the identification of *BRCA1* genomic rearrangements in 12 independent families. Noteworthy, half of the probands carry mutations that recur in more than one Italian family. Considering the whole spectrum of Italian *BRCA1* gene rearrangements identified thus far in consecutive patients, we estimate that alterations of this type account for 19% (95% CI: $0.11 < 0.19 < 0.28$) of the *BRCA1* mutation positive families. We conclude that the search for major genomic rearrangements is essential for an accurate and comprehensive *BRCA1* mutation detection strategy in Italy.

INTRODUCTION

Since the cloning of the two major breast cancer genes, *BRCA1* and *BRCA2*, screening for pathogenic mutations has largely failed to meet the expectation of identifying a molecular defect in most of the families genetically linked to the genes (Ford et al., 1998). The reasons for this inconsistency are likely to involve multiple factors. Among them is the fact that the occurrence of major genomic rearrangements that inactivate the two genes has broadly been underestimated until recently, since the commonly used mutation detection strategies are not specifically devised for this purpose. While a few genomic rearrangements have been identified in the *BRCA2* gene (Tournier et al., 2004; Agata et al., 2005; Mazoyer, 2005), a growing number of genomic alterations disrupting the *BRCA1* gene have been described in the last eight years (Mazoyer, 2005); nonetheless, an accurate estimate of their prevalence in different populations is still lacking. With the exception of a few studies specifically investigating a small number of recurrent *BRCA1* genomic rearrangements (*BRCA1* Exon 13 Duplication Screening Group, 2000; Hendrickson et al., 2005), systematic screenings of sufficiently large sample cohorts have been limited by the cumbersome technical approaches typically used for the search of major genomic rearrangements. Accordingly, the vast majority of the *BRCA1* genomic alterations described thus far were identified in

Supported by: Ministero dell'Istruzione dell'Università e della Ricerca, FIRB '01, PRIN '03; Lega Italiana per la Lotta contro i Tumori; Fondazione Cassa di Risparmio di Padova e Rovigo; Alleanza Contro il Cancro; Associazione Angela Serra per la Ricerca sul Cancro and Regione Veneto.

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Received 10 February 2006; Accepted 24 April 2006
DOI 10.1002/gcc.20342
Published online 19 May 2006 in Wiley InterScience (www.interscience.wiley.com).

studies on small, highly selected, sample series, thus leading to barely significant prevalence estimates.

The heterogeneous, worldwide distribution of *BRCA1* and *BRCA2* mutations across different ethnic groups likely represents an additional confounding factor. Population-specific mutational spectra have been described for both *BRCA1* and *BRCA2* point mutations, and, in some instances, are strongly influenced by founder effects that can even modify the overall mutation frequency (Neuhausen, 1999). The same genetic forces are likely to modify the distribution of genomic rearrangements in the two genes. Accordingly, a few founder *BRCA1* genomic rearrangements were described in more than one population (Mazoyer, 2005). For instance, two different genomic rearrangements of the *BRCA1* gene account for 23% of the Dutch mutation positive families (Hogervorst et al., 2003). On the other hand, ancient mutations such as the *BRCA1* founder exon 13 duplication display a worldwide distribution in populations of Celtic origin (*BRCA1* Exon 13 Duplication Screening Group, 2000).

Altogether, these observations imply that (a) the precise definition of the contribution of *BRCA1* and *BRCA2* genomic rearrangements to hereditary breast and ovarian cancer must be determined for every population and (b) it has to be estimated on a sufficiently large sample cohort.

By the analysis of a small subset of 27 highly selected families, we have previously shown that genomic rearrangements of the *BRCA1* gene play a role in cancer predisposition in families with breast and ovarian cancer patients (Montagna et al., 2003). To evaluate the prevalence and spectrum of alterations of this type in the Italian population, we now have used the Multiple Ligation-dependent Probe Amplification (MLPA) technology to analyze a cohort of 653 affected probands without identifiable *BRCA1* or *BRCA2* point mutations, including patients from four different Italian centers.

MATERIALS AND METHODS

Families Recruitment

The probands analyzed in this study were recruited from four different Italian Institutions, three located in the North (Aviano, Padua and Modena) and one in the South (Catanzaro) of the country. Although about 90% of the families were recruited from the Northern centers, these also included patients from Southern and Central Italy. Inclusion criteria were rather flexible and fulfilled at least one of the previously reported criteria (Santarosa et al., 1998). In addition, single cases were also included when affected by both breast and ovarian cancer, diagnosed with bilateral breast cancer, diagnosed before age 30, or if male breast cancer patients. Blood samples were obtained from each affected proband after informed consent on the aims of the study.

BRCA1 and *BRCA2* Mutation Analysis

DNA was extracted by standard phenol–chloroform techniques or by means of commercial DNA extraction kits from either whole blood or from EBV immortalized lymphoblastoid cell lines established according to standard procedures (Menin et al., 1996). Pathogenic point mutations in *BRCA1* and *BRCA2* genes were excluded by DHPLC and direct sequencing as well as SSCP and PTT in a minority of cases. The screening for major genomic rearrangements was carried out in two of the four centers by means of the MLPA methodology (Schouten et al., 2002) using the P002 and P087 kits (MRC Holland, Amsterdam, the Netherlands), according to the manufacturer's instructions. Variations in peak height were evaluated by comparing each sample with a normal control and/or by comparison of the relative area of each peak to the average value obtained from normal control samples of the same experiment.

Breakpoint Characterization

Long-range PCR was performed with the Expand Long Template PCR System (Roche Indianapolis, IN) according to the suggestions provided by the manufacturer. RT-PCR analysis of the *BRCA1* transcripts was carried out using the Superscript^{TM} RT RnaseH^{-} reverse transcriptase (Invitrogen, Carlsbad, CA) on RNA isolated using the RNAzol reagent (Tel-Test, Friendwood, TX). Primer pairs used to amplify the breakpoint region from cDNA or genomic DNA are available upon request. All sequence reactions were performed with the DNA sequencing kit (Applied Biosystems, Foster City, CA) and subjected to capillary electrophoresis with the ABI Prism-310 Genetic Analyser (Applied Biosystems). When necessary, sequencing reactions were preceded by cloning of PCR products using the TOPO TA cloning Kit (Invitrogen).

Allelotyping of the *BRCA1* Locus

Genotyping of the microsatellites *D17S855*, *D17S1322*, *D17S1323*, and *D17S1327* was achieved by independently amplifying each marker by PCR using FAM-labeled forward primers followed by visualization of the PCR products by capillary gel electrophoresis using the ABI Prism-310 Genetic Analyser (Applied Biosystems).

TABLE 1. Characteristics of *BRCA1* Genomic Rearrangements in 12 Patients Belonging to Breast and/or Ovarian Cancer Families

Family number	Rearranged exons by MLPA (P002)	Breakpoint coordinates[a] (L78833)	Mutation effect[b]	Confirmation by alternative MLPA kit (P087)
AV-3	del 1a-2	ND	*No transcript*	+
B.M.	del 1a-2	ND	*No transcript*	+
M.M.	del 5	ND	*In frame deletion of 26 AA*[c]	+
AV-181	del 5–7	ND	*Premature termination at codon 60*	+
AV-105	del 5–8	ND	*Premature termination at codon 47*	+
AV-258	del 9–19	g.29197_65577del36381	del ex. 9–19 ter 184	+
GUA	del 16–20	g.56990_75331del18342	del ex. 16–20 ter 1628	+
B361	del 17	g.58759_61875del3117	del ex. 17 ter 1672	+
B541	del 17	g.58759_61875del3117	del ex. 17 ter 1672	+
AV-142	dup 20	g.67058_75763dup8706	In frame dup ex. 20 (28AA)	+
AV-110	del 23	ND	*Unpredictable*	del 23–24
AV-267	del 23	ND	*Unpredictable*	del 23–24

[a]ND: not determined.
[b]Mutation effects reported in italic were predicted on the bases of the genomic alterations and were not experimentally confirmed.
[c]AA: aminoacid.

RESULTS

Among the 653 probands analyzed for *BRCA1* gene rearrangements, 12 led to results consistent with the presence of a genomic alteration involving one or more exons of the *BRCA1* gene (Table 1). Except for one putative duplication, all the other alterations were compatible with deletions and were consistently reproduced by at least one confirmatory experiment. Half of the alterations involved at least two contiguous exons and, therefore, were unlikely to represent false positive cases. Nonetheless, based on our previous experience with the MLPA technique, we sought to obtain proof of the genetic alteration from at least one additional independent and unrelated experiment.

The deletion of exons 16–20 in patient GUA was confirmed by the cloning of the breakpoint region and shown to be due to an unequal recombination between two homologous Alu sequences located in intron 15 and 20. The analysis of the mutant transcript further confirmed the absence of exons 16–20 that caused the formation of a premature stop codon at amino acid 1628.

Breakpoint characterization was also performed in proband AV-142, carrying a putative duplication of exon 20, employing a sort of "inverse PCR" approach. After unsuccessful attempts to amplify the whole region between exons 19 and 20, we performed a long-range PCR reaction using an "inverted" pairs of primers mapping in exon 20 (the forward primer was located two bases downstream of the reverse one) (Fig. 1), and selectively amplified a ~9-kb fragment containing the genomic region located between the two copies of exon 20. The breakpoint containing region was narrowed down by walking within intron 19 with different reverse primers finally leading to identification of the recombining Alu sequences (Fig. 1). At the transcript level, the exon 20 "inverted" primer pair allowed the amplification of a PCR fragment containing two tandem copies of exon 20, which would result in preservation of the correct reading frame and duplication of 28 aminoacids.

The putative deletions of exons 17 (probands B361 and B541) and 9–19 (proband AV-258) occurred in exons previously involved in other Italian families (Montagna et al., 1999; Montagna et al., 2003); therefore, breakpoint-specific primer pairs were used to investigate the identity of these alterations. All three cases gave a positive result and identical breakpoints were confirmed among patients with the same deletions (data not shown).

While the study was in progress, an alternative *BRCA1*-specific MLPA kit (P087), employing a completely independent *BRCA1* exon-specific probe set became available. The analysis of the remaining samples with the new probes not only confirmed all rearrangements, but suggested that the deletion of exon 23 in families AV-110 and AV-267 apparently extended to exon 24 as well (Table 1). At the same time we became aware of a cross-hybridization of P002 exon 24 probes to an unrelated sequence previously mapped to chromosome band 4q28 (Stec et al., 2001), as declared by the MLPA manufacturer. Therefore, in both patients, these deletions very likely included exons 23 and 24. Concordant results were obtained in an affected relative of family AV-110 who inherited the deletion.

Considering all Italian genomic rearrangements reported either in this or in previous studies (Montagna,

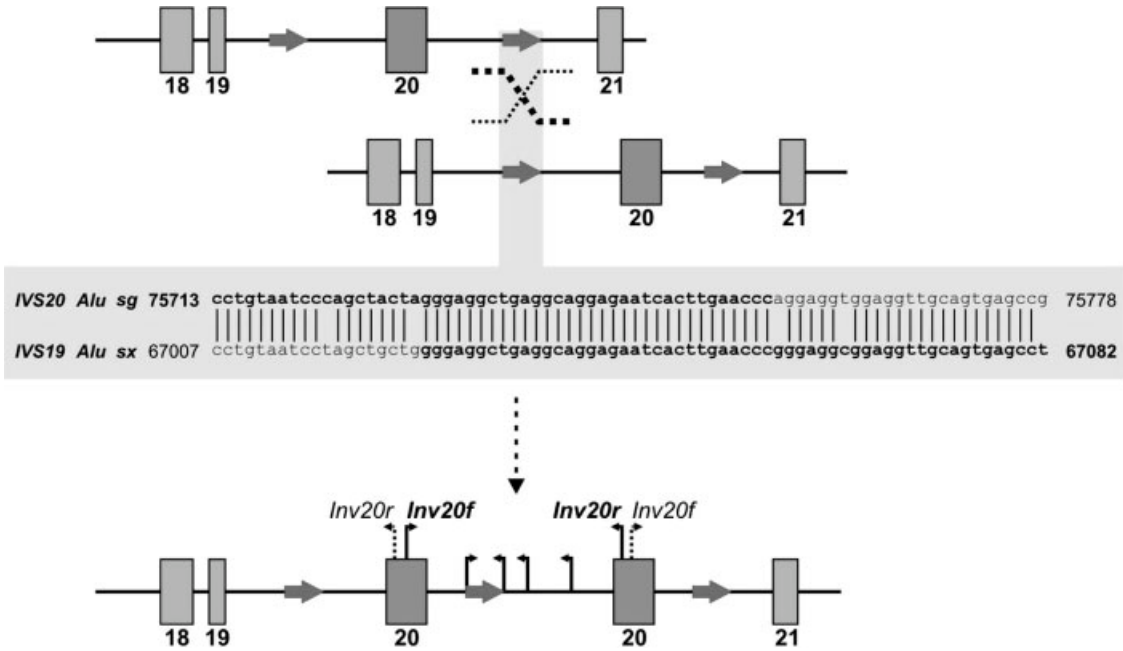

Figure 1. Location and sequence comparison of the Alu repeats involved in the recombination leading to the exon 20-duplicated allele of patient AV-142. The putative recombination event mediated by Alu repeats (horizontal arrows) is shown in the top figure. Homology between the recombining sequences across the breakpoint is outlined in the central box with the rearranged sequence in bold. Location of the primers used for the characterization of the duplicated allele (bottom figure) is indicated by vertical arrows. Distances and exon dimensions are not to scale.

et al., 1999; Montagna et al., 2003) four mutations (deletions of exons 1a–2, 17, 23–24, and 9–19) occurred in more than one independent family, thus suggesting a common ancestral origin (Table 2). To investigate this hypothesis, the microsatellite markers *D17S855*, *D17S1322*, *D17S1323*, and *D17S1327* were analyzed in all of the families carrying the same genetic alterations. Sharing of the same allelic combinations was demonstrated for the two probands with the exon 9–19 deletion as well as for the four carriers of exon 17 deletion; the allelic phase was determined for probands B74 and B361 where additional family members were available. Unexpectedly, genotypes compatible with common ancestry were not observed in patients with the deletion of exons 23–24 as well as in the four probands carrying the 1a–2 deletion.

Among all affected family members, six had bilateral breast cancer and nine were affected by breast and ovarian cancer (Table 2). Although both these features are good predictors of a *BRCA1* mutation in our series of samples, the mean number of ovarian as well as bilateral breast cancers did not differ significantly from that observed in the survey of 104 Italian pedigrees with *BRCA1* point mutations (Aretini et al., 2003). Similarly, the mean age of cancer diagnosis among probands (42 ± 2.3 and 54 ± 3.4 for breast and ovarian cancer, respectively) as well as the number and type of additional cancers were in line with those previously reported for carriers of *BRCA1* point mutations (Aretini et al., 2003). As expected, the majority of genomic rearrangements occurred in families with high probability of *BRCA1/2* mutations as shown by the high scores obtained with the Italian version of the BRCAPRO software (Marroni et al., 2004) (Table 2). No specific phenotypes were apparent from the analysis of the 18 histological reports available from 14 of the 19 probands. In particular, most of the breast tumors were ductal infiltrating cancers. The only exception was a rare form of neuroendocrine breast cancer in proband B.M. who carried the 1a–2 deletion. Whether this feature could be the result of co-deletion of *BRCA1*-contiguous genes remains to be evaluated. Available histopathologic data of probands' breast cancers confirmed some of the hallmarks of *BRCA1*-associated tumors, such as poor differentiation (histologic grades ranging from G2 in a few cases to G3 in most tumors), absence of estrogen and progesterone receptors expression (all but one tumors), and positivity for proliferation markers (Lakhani et al., 1998; Lakhani et al., 2002). Most ovarian cancer probands were affected by the serous variant of the tumor and only two by the endometrioid subtype. Overall, tumor histological grades ranged from moderately to poorly differentiated features.

TABLE 2. Cancer Histories of 19 Italian Breast and/or Ovarian Cancer Families with Genomic Rearrangements of the *BRCA1* Gene

Family number	Genomic rearrangement	Number of breast cancers[a] (age at diagnosis)	Number of ovarian cancers[a] (age at diagnosis)	Other tumors in the family	Prior probability (BRCAPRO)
B306	del 1a-2	1 (79)	1 (**51**)	–	0.340
B319	del 1a-2	2 (**45***, 41)	2 (**56***, 49)	–	0.767
AV-3	del 1a-2	0	2 (**43**, 30)	–	0.630
B.M.	del 1a-2	3 (40, 45, **57**)	1 (51)	–	0.581
M.M.	Del 5	2 (**62***, 80)	1 (**71***)	Uterus	0.187
AV-181	del 5–7	4 (**40**, 54/58, 89)	0	Larynx, uterus	0.176
AV-105	del 5–8	1 (32)	2 (**43**,62)	Lung	0.552
B212	del 9–19	5 (**37**, 39, 68, 43, 60)	1 (40)	NHL	0.707
AV-258	del 9–19	4 (**45**, 37, 50, 52)	0	–	0.853
GUA	del 16–20	6 (**27/37**, 32, 33, 40, 50, 60)	0	NHL, prostate (2)	0.996
B058	del 17	6 (**32**, 29, 49/50, 50, 44)	0	Larynx	0.927
B074	del 17	2 (**33**, 50*)	2 (52*, 46)	Colon	0.955
B361	del 17	2 (**48/64***)	1 (**65***)	–	0.559
B541	del 17	3 (**34**, 28, 62)	0	–	0.766
B242	del 18–19	8 (**40***[1], 30, 42, 50, 36*[2], 50/51*[3], 67)	3 (**50***[1], 44*[2], 73*[3])	–	0.997
B333	del 20	2 (**39**, 29)	1 (60)	–	0.846
AV-142	dup 20	3 (**32**, 55, 74)	0	Lung	0.130
AV-267	del 23–24	3 (**37**, 29/42)	0	–	0.653
AV-110	del 23–24	3 (**49***[1], 40*[2], 72)	3 (**53***[1], 45, 47*[2])	Prostate, thyroid, colon (2)	0.231

[a]Probands' tumors characteristics are indicated in bold; asterisks and apical numbers mark ages of patients with both breast and ovarian cancer; ages at diagnosis of bilateral breast cancer are separated by /.

DISCUSSION

Although large deletions and duplications of the *BRCA1* gene have been identified in many populations, clear indications for the screening of alterations of this type in breast/ovarian cancer families are currently not available for many countries, since studies based on sufficiently large sample cohorts are very rare. Here we report data on a large number of affected Italian probands, without detectable *BRCA1* and *BRCA2* point mutations, among whom 12 new cases carrying a *BRCA1* rearrangement were identified by employing the MLPA technique.

All 12 alterations very likely represent clinically relevant mutations that impair the BRCA1 protein function. More specifically, while deletions of the first exons (1a–2) likely affect the production and/or stability of the transcript, most of the other mutations lead to truncated proteins of variable sizes. The two in-frame alterations (deletion of exon 5 and duplication of exon 20) involve well conserved BRCA1 protein motifs. In particular, exon 5 includes cysteine residues of the RING domain frequently targeted by pathogenic point mutations. Differently, the 28 aminoacids encoded by exon 20 are part of the first BRCT (*BR*CA1 *C-t*erminal) domain (six aminoacids) as well as most of the "linker region" before the second BRCT. Evidences in favor of a crucial role of these aminoacids come from a number of observations. In particular (a) specific portions of the linker region, encoded by exon 20, are part of the interface resulting from packing of the two BRCT domains (Glover et al., 2004); (b) at least four missense substitutions within this region were identified in multiple breast/ovarian cancer families and shown to destabilize the protein fold as well as to impair ability to bind phospho-serine peptides that mediate interaction between BRCT and its protein partners (Williams et al., 2004); (c) in-frame deletion of these 28 aminoacids has been reported as the only pathogenic mutation in at least four different breast/ovarian cancer families worldwide (Belogianni et al., 2004; Mazoyer, 2005; Walsh et al., 2006).

With the exception of the exon 1a–2 deletion, none of the described alterations have been previously reported in other populations (Mazoyer, 2005), thus suggesting a country-specific mutational spectrum. Notably, taking into account all Italian families with *BRCA1* rearrangements in our series of samples, 12 of 19 cases carried mutations that recur in at least another family. Among probands with the same rearrangement, unshared inheritance of microsatellite alleles spanning the *BRCA1* locus was observed only for carriers of deletions involving exons 1a–2 or 23–24. Exon 1a–2 deletion has been identified in at least four additional families worldwide

(Mazoyer, 2005) and the absence of a common allelic combination among Italian patients is consistent with the idea that unequal recombination between *BRCA1* gene and its pseudogene may cause a hot spot for this specific type of *BRCA1* rearrangement (Puget et al., 2002). In contrast, deletion of exons 23–24 was identified in two independent families residing in the same geographical region and was never detected elsewhere in the world. The deviation from a common allelic combination was limited to a single allele of the intragenic dinucleotide *D17S1323* marker, which differed by two nucleotides from the expected allele size and could be explained by a mutation or recombination event occurred subsequently to the rearrangement.

Exon 17 deletion was identified in four independent pedigrees and represents the most frequent Italian rearrangement and the second most frequent *BRCA1* mutation in North-East Italy. This mutation was not observed in the screening of 982 breast and breast/ovarian cancer families from North America (Hendrickson et al., 2005) confirming its specificity to the Italian ethnicity. Importantly, the identification of recurrent genomic rearrangements has relevant implications for the design of accurate and sensitive diagnostic procedures. For instance, diagnostic PCR reactions can be easily designed to selectively amplify small DNA fragments that include the rearrangements breakpoints. Several rearrangements could then be simultaneously screened for by multiplex PCR reactions, making this analysis applicable even in non specialized laboratories.

Considering all probands of our series, the *BRCA1* rearrangements identified thus far account for ~2% of the breast and/or ovarian cancer families. Nonetheless, this figure (i.e., frequency of the rearrangements in all families) is rather unsuitable for comparison of different populations or different sample cohorts even within the same country as family selection criteria largely vary in different studies. Since no specific phenotypes were apparent when considering either families with the same rearrangement or all 19 cases as a whole, we believe that the contribution of genomic rearrangements to hereditary breast and ovarian cancer would be better represented by the percentage of the total number of *BRCA1* mutations; this would avoid misunderstandings due to the different criteria used for families recruitment.

To calculate the proportion of mutations due to large *BRCA1* gene rearrangements, we excluded the 99 nonconsecutive cases, among whom were three of the 19 rearrangement positive families reported in Table 2, as they represented only a subset of the mutation negative families. The remaining patients, recruited from two of the centers, were consecutive cases after the exclusion of 70 carriers of a *BRCA1* point mutation and 82 carriers of a *BRCA2* mutation. We therefore estimate that large deletions or duplications of the *BRCA1* gene account for 19% (16/86) (95% CI: $0.11 < 0.19 < 0.28$) of the *BRCA1* mutational spectrum. This fraction is lower than that previously hypothesized (Montagna et al., 2003), yet it still predicts two genomic rearrangements for every 10 *BRCA1* mutations. Whether a similar proportion will be accounted for by major rearrangements of the *BRCA2* gene is presently not clear. Available data apparently suggest a much less relevant contribution of alterations of this type to the *BRCA2* mutational spectrum (Tournier et al., 2004; Agata et al., 2005; Mazoyer, 2005). We therefore conclude that, at present, at least screening for *BRCA1* rearrangements should be included into Italian diagnostic screening procedures.

ACKNOWLEDGMENTS

We thank D. Zullato and M. Quaggio for their expert technical assistance, P. Gallo and A. Azzalini for artwork, and C. Case for help in preparing the manuscript. S. Agata is a fellow of the "Accademia Nazionale dei Lincei."

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