Characterization of an Italian Founder Mutation in the RING-Finger Domain of BRCA1

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Abstract

The identification of founder mutations in cancer predisposing genes is important to improve risk assessment in geographically defined populations, since it may provide specific targets resulting in cost-effective genetic testing. Here, we report the characterization of the BRCA1 c.190T>C (p.Cys64Arg) mutation, mapped to the RING-finger domain coding region, that we detected in 43 hereditary breast/ovarian cancer (HBOC) families, for the large part originating from the province of Bergamo (Northern Italy). Haplotype analysis was performed in 21 families, and led to the identification of a shared haplotype extending over three BRCA1-associated marker loci (0.4 cM). Using the DMLE+2.2 software program and regional population demographic data, we were able to estimate the age of the mutation to vary between 3,100 and 3,350 years old. Functional characterization of the mutation was carried out at both transcript and protein level. Reverse transcriptase-PCR analysis on lymphoblastoid cells revealed expression of full length mRNA from the mutant allele. A green fluorescent protein (GFP)-fragment reassembly assay showed that the p.Cys64Arg substitution prevents the binding of the BRCA1 protein to the interacting protein BARD1, in a similar way as proven deleterious mutations in the RING-domain. Overall, 55 of 83 (66%) female mutation carriers had a diagnosis of breast and/or ovarian cancer. Our observations indicate that the BRCA1 c.190T>C is a pathogenic founder mutation present in the Italian population. Further analyses will evaluate whether screening for this mutation can be suggested as an effective strategy for the rapid identification of at-risk individuals in the Bergamo area.

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Introduc
Haplotyping and estimate of mutation age

A total of 18 families, including 13 mutation carriers and 5 non-carriers, were genotyped at the BRCA loci previously described [12]. The MLDE22 software program (URL: http://www.dmle.org [13]) was used to establish the least number of possible recombinations. The proportion of sampled mutation-carrying chromosomes was estimated using the program MLDE22 [14], assuming a time interval of 52 years per generation. The value for population growth rates per generation (r) was estimated as 0.015, based on available demographic data (Italian National Statistics Institute, ISTAT: URL: http://www.istat.it). The proportion of sampled mutation-carrying chromosomes was estimated as 0.015, based on available demographic data (Italian National Statistics Institute, ISTAT: URL: http://www.istat.it). The proportion of sampled mutation-carrying chromosomes was estimated as 0.015, based on available demographic data (Italian National Statistics Institute, ISTAT: URL: http://www.istat.it).

Statistical analysis

The ratios of the peak areas of the BRCA1 and BRCA2 genes in different samples were compared by Student’s t-test using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). The p-values were calculated using the Student’s t-test.

Materials and Methods

Bacterial and mammalian expression vectors, including anti-parallel leucine zipper motifs, were created as described [15]. The plasmids were sequenced using the QuikChange XL Site-directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. Recombinant clones were obtained by direct XhoI digestion. The plasmid DNA was purified and reverse transcribed into cDNA as described [16]. The fluorescent PCR amplification products were run on a 3130 Genetic Analyzer (Applied Biosystems) using the GeneScan 500 ROX Size Standard (Applied Biosystems) as internal marker. The molecular weight of the peaks was confirmed by sequencing.

A Novel BRCA1 Italian Founder Mutation

Considering the prevalence of BRCA1 mutations in HBOC families, a study was conducted to identify and characterize a novel founder mutation in the Italian population. The study included 43 apparently unrelated families carrying BRCA1 mutations, identifying the c.190T>C mutation (p.Asp67Tyr) in exon 5 of the BRCA1 gene. The mutation was found to segregate with the disease in the Italian HBOC families, and was subsequently used for mutation age estimation.

Discussion

The identification of a novel founder mutation in the Italian population highlights the importance of genetic testing in HBOC families. The mutation, p.Asp67Tyr, is likely to be a significant contributor to the risk of breast and ovarian cancer in this population. Further studies are needed to confirm the prevalence of this mutation and to evaluate its impact on the disease risk.

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Fondazione IRCCS Istituto Nazionale dei Tumori (INT) and Istituto Ortopedico Rizzoli (IOR). Genetic testing and for the use of their biological samples for research and provided a written informed consent for BRCA gene mutation testing. This study was approved by the ethical committee of the Fondazione IRCCS Istituto Nazionale dei Tumori (INT) of Milan. A Novel BRCA1 Italian Founder Mutation
Purification of the reassembled GFP complexes
The H₆-NfrGFP-Z/Z-CfrGFP-HA and H₆-NfrGFP-BARD1/BRCA1-CfrGFP-HA (both as wild-type or mutant forms) complexes were purified from the soluble fraction of co-transformed E. coli strain BL21-(DE3) by Immobilized Metal Affinity Chromatography (IMAC) using nickel nitrilotriacetic (Ni-NTA) agarose resin (QIAGEN), following the protocol described [15]. The protein complexes were subjected to 13% SDS-PAGE and visualized by Western blotting using a polyclonal anti-GFP antibody (#600-101-215; Rockland). Unpurified cell lysates from induced E. coli BL21-(DE3) bacteria were similarly resolved and visualized to detect expression levels of both NfrGFP-BARD1 and CfrGFP-BRCA1 recombinant proteins.

Clinical and pathological characteristics
Clinical and pathological data of affected mutation carriers were collected at genetic counseling and from medical records. Loss of heterozygosity (LOH) at BRCA1 locus in tumor DNA was performed as previously described [16].

Results
Geographical distribution and frequency of the BRCA1 c.190T>C families
The birth places of the probands of the 43 BRCA1 c.190T>C positive families are shown in Figure 1. The majority were from the city of Bergamo and its province (n = 23) or from neighboring provinces of the Lombardy region (n = 16). The frequencies of c.190T>C positive families on the total number of families tested for BRCA1 and BRCA2 mutations and on the total number of BRCA-positive families were 8.7% and 30.2%, respectively, in cases recruited in Bergamo and 0.8% and 3.2%, respectively, in cases recruited in Milan (Table 1).

Figure 1. Geographical distribution of BRCA1 c.190T>C (p.Cys64Arg) mutation carriers. Symbol (‘*’) indicates the birth places of index case from families segregating the mutation. doi:10.1371/journal.pone.0086924.g001

Haplotype analysis
Fragment analysis of 6 microsatellite marker loci intragenic and flanking BRCA1 identified a shared haplotype extending over 3 marker loci (0.4 cM) in carriers of the BRCA1 c.190T>C from 20 families for which DNA of more than one member was available (Figure 2). In one additional mutated family with only one individual available for the analysis, the observed genotypes were compatible with the shared haplotype.

Age estimate of the BRCA1 c.190T>C mutation
The DMLE+2.2 program was used to estimate the age of the BRCA1 c.190T>C mutation based on haplotype data from mutation carriers and non carriers.

Three separate analyses were performed, using a population growth rate per generation of 0.052 and different estimates of the proportion of sampled mutation-carrying chromosomes (0.015, 0.01 and 0.005). The resulting age estimates were 124 generations (95% credible set: 79–170), 130 generations (95% credible set: 91–170) and 134 generations (95% credible set: 97–171), respectively (data not shown). Assuming an interval of 25 years per generation, this corresponds to the mutation being 3,100, 3,250 and 3,350 years old, respectively. Thus, age estimates were only slightly affected by the actual proportion of sampled mutation-carrying chromosomes.

Evaluation of the effect of the BRCA1 c.190T>C variant on mRNA transcripts
The BRCA1 c.190T>C variant is mapped to an alternatively used donor splice site. The usage of this site leads to the synthesis of a naturally occurring isoform missing 22 bp at the 3'-end of exon 5 (Δexon5q) [17,18]. To evaluate the putative effect of the mutation on Δexon5q and full-length transcription levels, the cDNA region encompassing the mutation site was amplified and analyzed by capillary electrophoresis. The ratio between the peak areas of the Δexon5q and the full-length isoforms in LCLs carrying the c.190T>C, cultured in the presence and in the absence of
cycloheximide, was calculated and compared with those of wild-type LCLs (Figure 3). The LCL carrying the \textit{BRCA1} c.212G>A mutation previously reported to up-regulate the $\Delta$exon5q isoform [14] was used as internal control. A significant decrease in the ratio of the $\Delta$exon5q vs. full length expression levels was observed in the c.190T>C LCLs compared to normal controls (0.45, 95\% CI = 0.44–0.46 and 0.52, 95\%, CI = 0.50–0.54, for the CHX-untreated and treated samples, respectively). Sequence analyses detected the presence of the mutation in the full length cDNA (data not shown). These results indicate that the c.190T>C mutation does not impair the synthesis of full-length mRNA and are consistent with a sustained expression of the mutated protein.

Evaluation of the effect of the c.190T>C mutation on the interaction of BRCA1 with BARD1

The BRCA1 protein displays an E3 ubiquitin ligase activity mediated by the interaction with BARD1 through its N-terminal RING-finger domain [19,20], where the c.190T>C (p.Cys64Arg) is located.

To assess whether the mutation affects the BRCA1/BARD1 complex formation, we carried out a GFP-fragment reassembly screening, a Bimolecular Fluorescence Complementation-based assay (BiFC) [21]. In this assay, the GFP is dissected into two fragments (NfrGFP and CfrGFP) that, when expressed together in \textit{E. coli} cells, do not spontaneously reassemble into a fluorescence

\begin{table}
\centering
\begin{tabular}{|l|l|l|l|}
\hline
Institution & No. identified & \% on total tested families* & \% on BRCA1/2 families \\
\hline
INT & 22 & 1.03 (22/2140) & 3.98 (22/553) \\
IEO & 4 & 0.39 (4/1013) & 1.54 (4/260) \\
INT,IEO & 26 & 0.82 (26/3153) & 3.20 (26/813) \\
AO-BG & 16 & 8.74 (16/183) & 30.19 (16/53) \\
\hline
\end{tabular}
\caption{Number and frequencies of \textit{BRCA1} c.190T>C positive families among those recruited at three Italian institutions and tested for BRCA mutations.}
\end{table}

*Intake criteria for BRCA testing are described in Manoukian et al [38].

\begin{figure}
\centering
\includegraphics[width=\columnwidth]{haplotype_tree.png}
\caption{Haplotype branching trees in families segregating the \textit{BRCA1} c.190T>C (p.Cys64Arg). The six short tandem repeat markers analyzed are shown together with their position in the Marshfield genetic map. Family haplotypes are indicated with the corresponding family ID codes. The most common haplotype is indicated in bold numbers.}
doi:10.1371/journal.pone.0086924.g002
\end{figure}
protein. However, if the two fragments of GFP are each individually fused to two interacting proteins, this interaction can mediate reassembly of the GFP in co-transformed bacteria with consequent cellular fluorescence [22].

Under inducing conditions (0.2% L-arabinose and 20 μM IPTG; Fig. 4A, left column), bright fluorescence was observed in bacterial cells co-expressing BARD1-NfrGFP together with BRCA1-wild type/CfrGFP or BRCA1-D67Y/CfrGFP carrying the variant p.Asp67Tyr, previously classified as clinically neutral [3] and the strong interacting anti-parallel Z-NfrGFP/Z-CfrGFP fusion peptides. No fluorescence was observed in bacteria co-expressing the following fusion peptides: non cognate BARD1-NfrGFP/CfrGFP-Z, BARD1-NfrGFP/BRCA1-C61G-CfrGFP carrying the p.Cys61Gly disease-causing mutation [23], and BARD1-NfrGFP/BRCA1-C64R-CfrGFP carrying the p.Cys64Arg mutation. In addition, IMAC purified reassembled

![Figure 3. Semi-quantitative fragment analysis of the Δexon5q isoform.](image)

The upper panel shows the capillary electrophoresis patterns of the cDNA fragments spanning BRCA1 exons 5 and 6 observed in LCLs from a BRCA1 wild type individual, and from carriers of the c.190T>C and c.212G>A, which causes the up-regulation of the Δexon5q transcript, mutations. The Δexon5q and full-length (FL) isoforms are indicated. The lower panel shows the ratio between the peak areas of the Δexon5q and full-length isoforms. The LCLs were cultured in the presence (dark grey bars) and in the absence (light grey bar) of cycloheximide. Control bars represent the average value observed in six wild-type LCLs. c.190T>C bars represent the average value observed in four mutant LCLs. The error bars represent standard deviation.

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complexes from the soluble fraction of co-transformed cells *E. coli* BL21-(DE3) were analyzed by Western blotting (Figure 4B). Using a polyclonal anti-GFP antibody, two strong bands corresponding to the components of the GFP reassembled complexes were detected in lysates of bacterial cells co-expressing Z-NfrGFP/ CfrGFP-Z and BARD1-NfrGFP together with BRCA1-wild type/ CfrGFP or BRCA1-Asp67Tyr. Much more reduced bands were observed in cell lysates co-expressing BARD1-NfrGFP and BRCA1-Cys61Gly/CfrGFP or BRCA1-Cys64Arg/CfrGFP. Since unassembled GFP fusion fragments are unfolded and less soluble [24], these results indicate GFP reassembly and, therefore, BRCA1/BARD1 binding for the BRCA1-wt and BRCA1-Asp67Tyr constructs, but not for the BRCA1-Cys61Gly and BRCA1-Cys64Arg constructs. These observations are consistent with those obtained by fluorescence complementation assay. Unpurified cell lysates from co-transformed *E. coli* BL21-(DE3) bacteria, were visualized by Western blotting with a polyclonal anti-GFP antibody, revealing that all the fusion peptides were expressed to a similar extent (Figure 4C). This demonstrates that the lack of co-purification of CfrGFP-BRCA1-HA fragments for the Cys61Gly and Cys64Arg constructs is attributable to the lack of binding to BARD1 and not to poor expression of the BRCA1 mutants.

**Clinical and pathological features of BRCA1 c.190T>C carriers**

Overall, a total of 86 ascertained mutation carriers and of 7 obligate carriers were identified in the families recruited in the study, including 83 females and 10 males. Among female carriers, 55 (66%) reported a diagnosis of breast carcinoma (n = 38), ovarian carcinoma (n = 12) or both breast and ovarian carcinomas (n = 5). Available clinical and pathological features of the affected female carriers are reported in Table 2 and 3. The median ages of breast cancer and ovarian cancer diagnosis were 39.6 and 48.2 years, respectively. The large majority of breast cancers were of high grade (23/28 = 82.1%). The prevalent histological type was ductal (32/38 = 84.2%), being the remaining cases mostly of the medullary type (4/38 = 10.5%). The frequencies of cancers positive for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/Neu) were 4/31 (12.9%), 6/31 (19.4%) and 3/22 (13.6%), respectively. The information for all the above tumor markers was available in 23

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**Figure 4. Detection of BRCA1/BARD1 interaction by GFP-fragment reassembly screening.** (a) Fluorescence was observed after 24 h of growth at 30°C followed by 2 days of incubation at RT. No fluorescence is observed under non-inducing condition (right column). [L-ara, L-arabinose; IPTG, Isopropyl-β-D-1-thiogalactopyranoside, IPTG]. (b) SDS-PAGE of purified, reassembled complexes by IMAC methods. The expected molecular masses are indicated on the left. [*Non-specific band. B°, H°-NfrGFPBARD1; Z°, H°-ZNfrGFP; Z°, ZCfrGFP-HA*.] (c) Expression of NfrGFP-BARD1 and CfrGFP-BRCA1 wild-type and mutant forms.

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**A Novel BRCA1 Italian Founder Mutation**
cases, and the majority (15 = 65.2%) displayed a triple-negative (TN) phenotype (lack of the expression of ER, PR and HER2/Neu). The frequencies of contralateral breast cancer were 8/18 (44.4%) and 12/15 (80%) 5 and 10 years after the first diagnosis of breast cancer, respectively. Three patients were diagnosed with additional cancers, including one malignant mixed mullerian tumor (MMMT) of the endometrium, one endometrial carcinoma, and one basal cell carcinoma. As for ovarian cancers, the large majority were of high grade (10/11 = 90.9%) and of the serous type (9/11 = 81.8%). Of the remaining female carriers, one reported a pheochromocytoma at 27 years, and 27 were cancer-free at the time of last contact (mean age = 41 years).

Three cancer diagnoses were reported in male carriers, one prostate carcinoma, one tumor of the central nervous system and one leukemia.

LOH at BRCA1 locus was investigated in two tumors, one breast carcinoma and the MMMT. In both cases, the loss of the constitutional wild-type allele was observed (data not shown).

### Discussion

The BRCA1 c.190T>C mutation was firstly described in a large Polish cancer family [25]. Subsequently, it was reported in an Italian family characterized by high prevalence of ovarian and breast cancer cases by Willems and co-authors [26]. These authors showed by molecular modeling that the mutation may induce profound modifications in the structure of the BRCA1 RING finger motif, without affecting the normal splicing pattern of the transcripts.

A total of 43 apparently unrelated Italian families carrying the same mutations were referred by four different Italian clinical and university centers. The observation that a large part (23/43 = 53%) were from the same geographic area, the province of Bergamo in Northern Italy, strongly suggested an origin of the mutation from a common ancestor. Consistently, the frequencies of c.190T>C families on the total number of those that underwent BRCA gene testing and of those carrying pathogenic BRCA1 and BRCA2 mutations were approximately 10-fold higher in cases recruited in Bergamo, around 83% of whom referred to be born in the local area, compared to those observed in families recruited in two large cancer centers in Milan, which attract patients from all over Italy. The analysis of microsatellite marker loci revealed a shared haplotype in 21 typed families, confirming the founder effect hypothesis. In addition, we estimated the origin of this mutation within a time interval ranging from 3100 to 3350 years ago.

Interestingly, the c.190T>C affects the same nucleotide of another mutation (c.190T>G) that strengthen an alternatively used donor splice site in exon 5 and also disrupts a putative exonic splicing enhancer motif. This leads to the loss of the natural donor splice site, resulting in the lack of full length expression and the up-regulation of the naturally occurring Δexon3q isoform, predicted to lead to the synthesis of a truncated protein [17,18]. In agreement with previous observations [26], no such effect was observed for the c.190T>G. Conversely, in the LCL carrying this mutation a reduction in the relative amount of Δexon3q compared to full length was observed. This finding and the detection of the c.190T>C in the full-length cDNA is consistent with a stable expression of the Cys64Arg mutation in protein carriers.

The BRCA1 p.Cys64Arg mutation is located to the gene region coding for the RING-finger motif of the protein. The RING-finger motif is defined by a conserved pattern of seven cysteine and one histidine residues arranged in an interleaved fashion forming two distinct Zn2+-binding sites termed Site I and Site II [27].

| Table 2. Clinical and pathological features of BRCA1 c.190T>C related breast cancer cases. |
|-----------------------------------------------|
| **Age at first diagnosis (years)** | n | % |
| <36 | 15 | 34.9 |
| 36 | 22 | 51.2 |
| >50 | 6 | 14.0 |
| **Median** | 39.6 |
| **Behaviour** |  |
| Invasive | 40 | 100 |
| In situ | - | - |
| **Histological types** |  |
| Ductal | 32 | 84.2 |
| Lobular | - | - |
| Medullary | 4 | 10.5 |
| Combineda | 1 | 2.6 |
| Other | 1 | 2.6 |
| **Histological grade** |  |
| 1 | 2 | 7.1 |
| 2 | 3 | 10.7 |
| 3 | 23 | 82.1 |
| **ER** |  |
| Positive | 4 | 12.9 |
| Negative | 27 | 87.1 |
| **PR** |  |
| Positive | 6 | 19.4 |
| Negative | 25 | 80.6 |
| Not available | 12 |
| **Her2/Neu** |  |
| Positive | 3 | 13.6 |
| Negative | 19 | 86.4 |
| **Triple Negative** |  |
| Yes | 15 | 65.2 |
| Not | 8 | 34.8 |
| Not available | 20 |

Approximately 10% of the clinically relevant mutations of BRCA1 currently reported in the Breast Cancer Information Core (BIC) database (URL: http://research.nhgri.nih.gov/bic/) map within the N-terminal 100 residues-RING domain, which contain the RING motif (residues 23–76). Functional analyses have shown that these mutations abrogate the ubiquitin-ligase activity of BRCA1 by interfering with both the heterodimerization between BRCA1 and BARD1 and the binding of E2 protein UbcH5c to the BRCA1/BARD1 complex [28,29].
Presently, the BRCA1 p.Cys64Arg mutation is reported in the BIC database as a variant of unknown clinical relevance. However, in silico analysis using SIFT (URL: http://sift.jcvi.org/), Polyphen-2 (URL: http://genetics.bwh.harvard.edu/pph2/) and Align-GVGD (URL: http://agvgd.iarc.fr/) bioinformatics tools unanimously predicted the BRCA1 p.Cys64Arg to be deleterious. This is consistent with the notion that Cys64Arg disrupts a critical cysteine residue required for the ubiquitin-ligase activity of BRCA1, through its binding to BRD1 [30]. In fact, the analysis of the three-dimensional Nuclear Magnetic Resonance (NMR) structure of the BRCA1/BRD1 heterodimer showed that p.Cys64Arg substitution determines a profound rearrangement of the BRCA1 39–41 amino acid residues, predicted to result into the impairment of the BRCA1/BRD1 interaction [26].

The result of our GFP-fragment reassembly assay provided experimental evidence that the p.Cys64Arg actually abrogates BRCA1/BRD1 binding as the proven disease causing mutation p.Cys61Gly [31], thus further supporting the pathogenic role of this variant. Interestingly, another mutation affecting the same amino acid residue (p.Cys64Gly) was previously reported to have a similar effect [32]. In addition, our study indicates that this functional approach might be applied to the assessment of the clinical significance of a number of variants in cancer-predisposing genes, implementing currently used strategies.

The median age of breast cancer diagnosis in carriers of the c.190T>C mutation (39.7 years) was similar to that observed in 425 breast cancer patients with other pathogenic BRCA1 mutations ascertained at the institutions participating in this study (40.1 years). Conversely, among c.190T>C carriers the median age of ovarian cancer onset (48.2 years) was lower than that observed in 192 BRCA1-positive ovarian cancer patients from the same institutions (51.9 years), suggesting a possible higher risk of ovarian cancer in carriers of the c.190T>C compared to carriers of other BRCA1 mutations.

Breast cancers arising in BRCA1 c.190T>C mutation carriers showed the peculiar histopathological features of the BRCA1-related breast tumor [33,34], with a propensity to be high grade invasive ductal or medullary carcinomas. In addition, the majority displayed a TN phenotype. The clinical relevance of the TN breast cancers is highlighted by the distinctive poor prognosis and no clear options for receptor targeted treatment [35,36]. In agreement with other reports on BRCA1 associated ovarian carcinomas [37,38], invasive epithelial cancer of serous histology was found to be the most common histological subtype among BRCA1 c.190T>C mutation carriers. Although these observations might be biased by the relative small number of ascertained individuals and by the preferential selection of high risk subjects referred to genetic testing, they are consistent (together with the findings that in two examined tumors the wild type BRCA1 allele was lost) with the c.190T>C behaving as other BRCA1 pathogenic variants.

In conclusion, our data show that the BRCA1 c.190 T>C missense mutation (p.Cys64Arg) is a founder mutation of clinical relevance recurrent in high-risk families from the province of Bergamo, accounting for a significant fraction (ca. 9%) of those ascertained at the local city hospital. Additional studies are needed to assess the actual proportion of carriers of this mutation in this district and its frequency in unselected breast/ovarian cancer patients. This will allow evaluating whether screening for the c.190T>C mutation can be suggested as a cost-effective strategy for the rapid identification of at-risk individuals in the Bergamo area. In addition, they will provide a measure of associated cancer risks, thus improving decision-making regarding clinical management of mutation carriers.

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Author Contributions

Conceived and designed the experiments: LC ALP MC CBR LP PR. Contributed to case selection by performing BRCA testing: VP LB. Performed the experiments: LC ALP MC CC CBR. Analyzed the data: Conceived and designed the experiments: LC ALP MC CBR LP PR.

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