Abnormality of the DNA double-strand-break checkpoint/repair genes, \textit{ATM}, \textit{BRCA1} and \textit{TP53}, in breast cancer is related to tumour grade

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The role of the DNA double-strand-break (DSB) checkpoint/repair genes, \textit{ATM}, \textit{BRCA1} and \textit{TP53}, in sporadic breast cancer requires clarification, since \textit{ATM} and \textit{BRCA1} mutations are rare in sporadic tumours. In an attempt to explain this phenomenon, we postulated that (i) in addition to genetic deletion, abnormal expression of DSB checkpoint/repair proteins might abolish the function of these genes and (ii) there might be a combined effect of individual defective genes during breast cancer pathogenesis. Using a largely homogenous group of 74 specimens of early-onset (≤35 years of age) infiltrating ductal carcinomas, we examined associations between pathological grade and genetic deletion and/or abnormal protein expression of \textit{ATM}, \textit{BRCA1} and \textit{TP53}. The results showed that high-grade tumours displayed a high frequency of loss of heterozygosity (LOH) at, and/or abnormal expression of, \textit{ATM}, \textit{BRCA1} and \textit{TP53}. Multigenetic analysis showed abnormalities in \textit{BRCA1} to be independently associated with high-grade tumours. \textit{ATM} and \textit{TP53} appeared to play an assistant role, abnormalities in these genes significantly increasing the possibility of poor differentiation in tumours with abnormalities in \textit{BRCA1}. Furthermore, a higher number of abnormalities (LOH or abnormal expression) in these three genes correlated with poor tumour differentiation. Thus, this study suggests that combined changes in several DSB checkpoint/repair genes belonging to a common functional pathway are associated with breast cancer pathogenesis.

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Tumorigenesis results from a series of genomic alterations that leads to progressive disorder of the normal mechanisms controlling cell growth, death and differentiation (Bishop, 1991). Our recent studies have demonstrated that the extent of DNA double-strand-break (DSB)-initiated genomic deletion in tumours is significantly increased in high-grade breast tumours (Shen \textit{et al}, 2000). On the basis of these findings, we hypothesised that \textit{ATM}, \textit{BRCA1} and \textit{TP53}, the critical genes in the DSB checkpoint/repair pathway, might play an important role during breast tumorigenesis, and that defects in these genes could result in poor tumour differentiation. A causal link between breast cancer development and mutation of \textit{ATM}, \textit{BRCA1} and \textit{TP53} has been found in familial breast cancer syndromes (Buchholz \textit{et al}, 1999). However, the probability of finding \textit{ATM} and \textit{BRCA1} mutations in sporadic breast cancer is low (Vorechovsky \textit{et al}, 1996; Bay \textit{et al}, 1998; Papa \textit{et al}, 1998). In contrast, genomic deletions at the loci harbouring these three genes are relatively frequent (Lo \textit{et al}, 1998; Rio \textit{et al}, 1998; Shen \textit{et al}, 2000). Genomic deletion represents one of the ‘two hits’ needed to inactivate tumour suppressor genes for cancer formation (Knudson, 1971). Recent studies have suggested that epigenetic mechanisms, manifested as abnormal protein expression, serve as the other hit, and are involved in abrogating the function of certain tumour suppressor genes (Jones and Baylin, 2002). In addition, as our understanding of cancer development extends beyond single-gene disorders to multigenetic disorders and aetiological pathway-wide abnormalities, it is tempting to speculate that the combined effect of several defective genes in a common antitumour pathway could lead to more poorly differentiated tumours. We therefore carried out the present study, using a highly homogenous study population and a multigenetic design, to examine the relationship between abnormalities (both genetic deletion and abnormal expression) in the most critical DSB checkpoint (\textit{ATM} and \textit{TP53}) and repair (\textit{BRCA1}) genes and tumour grade.

\textbf{MATERIALS AND METHODS}

\textbf{Patients and specimens}

The study subjects, 74 Chinese women with early-onset (≤35 years of age) breast cancer, were a subset of patients selected from our
ongoing hospital-based breast cancer cohort (Yang et al, 1997; Shen et al, 2000). Breast cancer in Taiwanese (Chinese) women is characterised by a low incidence (Yang et al, 1997), early tumour onset (Lo et al, 1998) and novel genomic alterations (Lou et al, 1997; Shen et al, 2000). Owing to the low incidence of breast cancer, which suggests an overall lower effect of common risk factors, and its homogenous genetic background, the Taiwanese population has certain advantages for studying the effects of genetic variations. In order to obtain an aetiologically homogenous group of study subjects, which should help in elucidating the causes of tumour, we restricted our study to histologically confirmed, primary infiltrating ductal carcinomas (IDCs). Direct causes of tumour, we restricted our study to histologically confirmed, primary infiltrating ductal carcinomas (IDCs). Direct sequencing showed that none of the patients had germline mutation of the BRCA1 gene. Furthermore, no patient had a family history of breast cancer or of Li–Fraumeni syndrome (hereditary, and associated with mutant TP53) in their first-degree relatives, and no case of germline mutations in the ATM gene has ever been identified in breast cancer families in Taiwan, so these tumours were most likely sporadic. On grading using the Scarff–Bloom–Richardson system (Le Doussal et al, 1989), the number of IDCs of each grade was found to be similar. Institutional review board-approved informed consent was obtained from each patient (Shen et al, 1997; Shen et al, 1998). All specimens were stored at −80°C until analysed.

Allelotyping PCR and definition of loss of heterozygosity (LOH)

A PCR-based method was used to detect loss of heterozygosity (LOH) at loci within, or close to, ATM, BRCA1 or TP53. The allelic status of these genes was determined using six microsatellite markers, two for each gene: D11S1816 (11q22–11q23), close to ATM, D11S2179 (intron 34 of ATM), D17S1322 (intron 19 of BRCA1), D17S1323 (intron 12 of BRCA1), TP53 (intron 1 of TP53) and D17S786 (17p13, close to TP53). The sequences of the primers were: D11S1816, forward 5'-ATTGTTGAAGCTAGGTGCTGG-3' and reverse 5'-AAAGAGATAAAAACAGATTCTGGATG-3'; D11S2179, forward 5'-TACAGAGTATCCAGACCCCTG-3' and reverse 5'-TCCTGTTCTAGTTGCTCTAGT-3'; D17S1322, forward 5'-CTAGCCTGGGAAACAAACAAG-3' and reverse 5'-GGAGGAGCTGATGGAAGCTG-3'; D17S1323, forward 5'-TTCGAGGTGTTTTGCTTCTG-3' and reverse 5'-AAGCAGGAATGGAAC-3'; D17S786, forward 5'-GCAGGTGGGCTCTTCTG-3' and reverse 5'-GGATTATTGGTG-3'. The PCR amplification was carried out using 100 ng of DNA from tumour tissue or peripheral blood, 0.4 U of Taq polymerase, 0.2 mm deoxynucleotides and 2.5 mm MgCl2 in a total reaction volume of 10 µL. The PCR conditions were 95°C for 12 min to activate Taq polymerase, followed by 40 cycles of denaturation (95°C, 45 s), annealing (55°C, 30 s) and extension (72°C, 45 s), the final elongation being performed at 72°C for 10 min. PCRs were run in a GeneAmp PCR 9600 thermocycler (PE Bioskysystems, Foster City, CA, USA). PCR amplifications omitting template DNA were included in each experiment as a control for contaminating DNA, and a housekeeping gene (β-actin) was used as an endogenous control. PCR products were electrophoresed on a 377 ABI PRISM sequencer, and the fluorescent signals from the different-sized alleles recorded and analysed using GENTYPER (version 2.1) and GENESCAN (version 3.1). For an informative marker (heterozygous for the two alleles in blood specimens), the locus was considered to display LOH when there was a four-fold or greater difference in the relative allele intensity ratio between the tumour DNA and normal DNA (Figure 1). Deletion of one allele of ATM, BRCA1 or TP53 was defined by either of the two markers showing LOH.

**Immunohistochemistry**

Tissue specimens were fixed in 4% neutral-buffered formaldehyde, embedded in paraffin and cut into 4 µm thick sections, which were deparaffinised for 3 × 5 min in xylene, rehydrated in graded alcohol and rinsed in Tris-buffered saline containing 0.1% Tween 20 (TBST). To improve antigen retrieval, de waxed sections were immersed in 0.001 M EDTA, pH 8.0, heated for 30 min in a pressure cooker (TAC-10KS, Tatung, Taiwan), cooled to room temperature for 15 min, then rinsed briefly in TBST. Endogenous peroxidase was blocked by incubation for 5 min at room temperature in 3.5% hydrogen peroxide in TBST, followed by a TBST wash for 5 min. Nonspecific binding of antibodies was blocked by incubation of the sections for 30 min at room temperature with normal rabbit serum (1:5 in TBST). After washes with TBST, primary mouse monoclonal antibodies were added and the sections incubated in a moist chamber for 1 h at room temperature. Following several

**Figure 1**  Allelotyping PCR to detect LOH of BRCA1 (trinucleotide marker D17S1322) in three representative breast tumours. The locus of the marker was considered to show LOH when a four-fold or greater difference was seen in the relative allele intensity ratio (allele 1 : allele 2) between the tumour and normal DNA. Tumour (A) showed LOH at this locus. Tumour (B) showed a relative allele intensity ratio for tumour and normal DNA of 1:12, and was considered not to show LOH at this marker. Tumour (C) was homozygous at this marker and was noninformative.
rinses, biotinylated secondary antibody was applied for 30 min at room temperature, then the sections were rinsed and peroxidase-linked streptavidin (Dako, Copenhagen, Denmark) was added for 20 min. The chromogen was developed with AEC (Dako, Copenhagen, Denmark) and the slides were lightly counterstained with haematoxylin to provide cellular detail. The primary monoclonal antibodies used were specific for ATM (undiluted, ATX 08, NeoMarkS, CA, USA), BRCA1 (1 : 125 dilution, SG11, Zymed, CA, USA) or TP53 (1 : 50 dilution, DO-7, DAKO, Copenhagen, Denmark). In negative controls, the primary antibody was replaced with TBST. In addition, the staining of infiltrating lymphocytes, stromal cells or adjacent normal epithelial cells within the tumours served as a normal control. Three pathologists independently evaluated the results semiquantitatively, and any discrepancy was resolved by joint review. Using previously reported criteria (Yoshikawa et al., 1999; Angele et al., 2000), we compared the tumours to the adjacent normal epithelium (in which most cells show positive staining for ATM and BRCA1) and classified those tumours with more than 25% of ATM- or BRCA1-negative tumour cells as showing reduced expression of these proteins, all others being classed as showing normal expression. Since mutant TP53 proteins generally have a longer half-life than wild-type TP53 protein, which leads to their nuclear accumulation (Finlay et al., 1988), tumours with more than 20% of nuclear TP53-positive tumour cells were classed as showing aberrant expression, all others being classed as showing normal expression. For simplicity, both the above cases ‘reduced’ expression of ATM and BRCA1 and ‘aberrant’ expression of TP53 are referred to as ‘abnormal’ expression in the main text.

Data analysis

The Mantel-extension $\chi^2$ test was used to examine the association between tumour grade and individual defective genes. Furthermore, to simultaneously examine all possible interactions between, and joint effects of, ATM, BRCA1 and TP53, multigenetic analysis based on logistic regression was performed. All statistical analyses were performed using SAS version 8.0 software (SAS Institute Inc., USA).

RESULTS

Genetic deletion and tumour grade

Using four intragenetic markers (D11S2179 for ATM, D17S1322 and D17S786 for TP53) and two adjacent markers (D11S1816 for ATM and D17S786 for TP53), our results showed that, after excluding noninformative tumours (six out of 74 at ATM, nine out of 74 at BRCA1 and 10/74 at TP53), the overall frequencies of LOH were 36.8% for ATM, 44.6% for BRCA1 and 53.1% for TP53 in these early-onset breast tumours. These frequencies are similar to those reported previously (Shen et al., 2000). As shown in Figure 2, even in grade I tumours, the LOH frequencies were 27.8% for ATM, 21.1% for BRCA1 and 30.0% for TP53. When an association between the frequency of LOH at individual genes and tumour grade (tumour differentiation) was examined using the Mantel-extension $\chi^2$ test for trends, BRCA1 and TP53 showed a trend to a significant increase in LOH frequency in more poorly differentiated tumours ($P = 0.003, P = 0.009$, respectively).

Abnormal protein expression and tumour grade

Immunohistochemical staining showed that the frequency of abnormal expression was 24.3% for ATM, 32.1% for BRCA1 and 28.4% for TP53, and that high-grade tumours tend to have an increased frequency of abnormal expression of these three proteins (Figure 3). When the frequency of abnormal expression of individual proteins was correlated with tumour grade using the Mantel-extension $\chi^2$ test for trends, there was a significant trend to an increase in abnormal BRCA1 expression with higher grade ($P = 0.036$) (Figure 3).

Coexistence of LOH and abnormal expression in the same gene

We then examined whether a combination of LOH and abnormal protein expression in the same gene was associated with poor differentiation. The results showed that, for ATM, combined LOH
and abnormal expression was not associated with tumour grade (Table 1). However, although the number of cases showing both LOH and abnormal expression in the same gene in each pathological grade was small, for BRCA1 and, to a lesser extent, TP53, these combined abnormalities were frequently seen in association with a high tumour grade ($P = 0.0003$, $P = 0.016$, respectively). These results are consistent with the idea that several important DSB checkpoint/repair genes could be inactivated by different mechanisms, and that tumours with LOH and/or abnormal expression in the same gene are more likely to exhibit poorer differentiation.

Defective BRCA1 is the most significant indicator, but defective ATM or TP53 provide an additional effect

The demonstration of a significant association between tumour grade and abnormality, either LOH or abnormal expression, at each DSB checkpoint/repair gene prompted us to ask which gene played the most critical role. Logistic regression analysis was performed to resolve this issue statistically. Since the age at the time of tumour onset is an important determinant for tumour grade (Jones and Laird, 1999; Bogdani et al, 2002), we included the patients‘ ages in this multivariate model. The analysis showed that LOH at BRCA1 ($P = 0.02$) and abnormal expression of BRCA1 protein ($P = 0.03$) were the only two factors significantly and independently associated with poor pathological grade (Table 2). This identification of the unique importance of BRCA1, however, did not exclude a joint effect of BRCA1 and other functionally related genes, and we therefore examined whether abnormalities in ATM or TP53 conferred an additional risk in cases in which BRCA1 was abnormal. The result of logistic regression showed a more significant association with high grade in tumours with abnormalities in BRCA1 and ATM and/or TP53 ($P = 0.001$) than in tumours with BRCA1 abnormalities alone ($P = 0.09$) (Table 3).

Multiple defective genes are seen in high-grade tumours

Support for our hypothesis that abnormalities in individual genes in the DSB checkpoint/repair pathway might act in combination came from the observation of a significant association between poor tumour differentiation and an increased number of these genes showing LOH. As shown in Table 4, 62% of grade I tumours showed no evidence of LOH at ATM, BRCA1 or TP53, whereas all three genes were intact in only 5% of grade III tumours ($P = 0.002$).

Furthermore, the results of the Mantel-extension $\chi^2$ test for trends confirmed the finding that an increased number of genes showing abnormal expression correlated with poor tumour differentiation ($P = 0.026$). However, since breast cancer always displays a high degree of intratumour heterogeneity in each individual tumour, it was possible that our finding of abnormal protein expression based on the overall abnormal frequency described above might fail to take into account intratumoral differences in protein expression. To exclude this possibility, we used serial sections to analyse the same region for the expression and subcellular location of the proteins of interest. Two typical examples are shown in Figure 4, in which a low-grade tumour displayed strong nuclear staining for ATM and BRCA1 and no TP53 accumulation, while a

Table 1 Genetic deletion (detected as loss of heterozygosity (LOH)) and abnormal expression (detected by immunohistochemical staining) of the DNA double-strand-break checkpoint/repair genes, ATM, BRCA1 and TP53, as a function of pathological grade in breast cancer

| Inactivating effects | No. of informative tumours | I | II | III | P-value \(^a\) for trend |
|----------------------|----------------------------|---|----|----|-----------------|
| ATM                  | 68                         | 13 (72.2%) | 12 (48.0%) | 8 (32.0%) | NS |
| No LOH plus normal expression | 33                         | 3 (16.7%) | 11 (44.0%) | 16 (64.0%) | |
| Either LOH or reduced expression | 30                         | 2 (11.1%) | 2 (8.0%) | 1 (4.0%) | |
| LOH plus reduced expression | 5                         | 12 (63.2%) | 7 (31.8%) | 3 (12.5%) | 0.0003 |
| BRCA1                | 65                         | 6 (31.6%) | 13 (59.1%) | 14 (58.3%) | |
| No LOH plus normal expression | 22                         | 1 (5.2%) | 2 (9.1%) | 7 (29.2%) | |
| Either LOH or reduced expression | 33                         | 12 (63.2%) | 7 (31.8%) | 3 (12.5%) | |
| LOH plus reduced expression | 10                        | 6 (31.6%) | 13 (59.1%) | 14 (58.3%) | |
| TP53                 | 64                         | 11 (55.0%) | 7 (30.4%) | 5 (23.8%) | 0.0161 |
| No LOH plus normal expression | 23                         | 7 (35.0%) | 13 (56.5%) | 9 (42.9%) | |
| Either LOH or aberrant expression | 29                        | 2 (10.0%) | 3 (13.1%) | 7 (33.3%) | |

\(^a\)P-values estimated using the Mantel-extension \(\chi^2\) chi-squared test.

Table 2 Logistic regression and odds ratio showing the relationship between pathological grade and either LOH or abnormal expression of ATM, BRCA1 or TP53

| Variable | $\beta$ regression coefficient | OR (95% CI) \(^b\) | P-value |
|----------|--------------------------------|--------------------|---------|
| LOH at ATM | -0.08                          | 0.93 (0.29–2.96) | NS      |
| LOH at BRCA1 | 1.48                          | 4.38 (1.25–15.41) | 0.02    |
| LOH at TP53 | 0.61                          | 1.84 (0.59–5.71) | NS      |
| Reduced ATM expression | -0.13 | 0.88 (0.19–4.04) | NS |
| Reduced BRCA1 expression | 1.35 | 3.84 (1.13–13.05) | 0.03 |
| Aberrant TP53 accumulation | 0.55 | 1.74 (0.46–6.57) | NS |

\(^b\)The odds ratio was adjusted for age (<30 years vs >30 years).

Table 3 Logistic regression analysis of breast tumour pathological grade as a function of abnormalities in BRCA1 alone or abnormalities in BRCA1 plus abnormalities in ATM and/or TP53

| Genetic deletion and/or protein abnormalities | $\beta$ regression coefficient | P-value \(^a,b\) |
|---------------------------------------------|--------------------------------|-----------------|
| BRCA1 alone \(^d\) | 2.43 | 0.090 |
| BRCA1 combined with ATM and/or TP53 \(^d\) | 3.90 | 0.001 |

\(^a\)The test was adjusted for age (<30 years vs >30 years). \(^b\)The reference group was defined as tumours with no LOH at, and no abnormal expression of, all three genes (ATM, BRCA1 and TP53). \(^d\)Abnormalities in BRCA1 (LOH and/or abnormal expression) alone. \(^e\)Abnormalities in BRCA1 combined with abnormalities in ATM and/or TP53 (either LOH or aberrant expression).
high-grade tumour displayed abnormal expression of all three proteins.

DISCUSSION

The rationale for a mutator contributing to the genomic instability that leads to cancer progression is that, instead of being a single-gene disease, cancer is caused by aberrations arising from a complex interconnecting network of multiple regulatory genes involved in normal growth control processes and the maintenance of genomic stability (Orr-Weaver and Weinberg, 1998; Loeb and Loeb, 2000). This rationale supports our approach of simultaneously examining the role of three important genes (ATM, BRCA1 and TP53) involved in the DSB checkpoint/repair pathway. Although abnormalities of individual DSB checkpoint/repair genes or proteins have been reported (Waha et al, 1998; Wilson et al, 1999; Angèle et al, 2000), almost all of these have been studied in isolation without considering the common pathway involved. In contrast, in the present study, using a largely homogeneous group of 74 early-onset IDCs and a multigenetic model, we have evaluated the tumorigenic contribution of LOH and/or abnormal expression of these genes. In terms of methodology, we selected intragenic markers and used laser capture microdissection for our LOH analysis to provide an unbiased estimate of the genetic status of these genes in cancer cells, and our careful examination of protein expression based on serial sections excluded the possibility of an effect of intratumour heterogeneity. The results (Figure 2) showed that loci harbouring ATM, BRCA1 or TP53 displayed a high frequency of LOH in low-grade tumours, suggesting that genetic deletion of these DSB checkpoint/repair genes frequently occurs during the early stage of breast tumour. This is consistent with the results of our previous study (Shen et al, 2000), which suggested that breast cancer progression is driven by DSB-initiated chromosomal instability. In addition, our immunohistochemical study (Figure 3) showed that high-grade tumours tended to have an increased frequency of abnormal expression of these three proteins and, in particular, that abnormal BRCA1 expression was related to poor differentiation (Figure 3), supporting an important contribution of DSB checkpoint/repair genes to breast cancer pathogenesis.

The present study also examined whether combined LOH and abnormal expression occurring at the same gene could contribute to the pathogenesis of breast cancer, and our findings for TP53 and BRCA1 seem to be in line with this hypothesis (Table 1). However, different mechanisms are involved in the effects of combined LOH and abnormal expression of TP53 and BRCA1. In sporadic cancers, LOH at the TP53 locus is usually accompanied by somatic mutation at TP53 (Yang et al, 1997; Kurose et al, 2002), leading to abnormal accumulation of this protein. Thus, a high frequency of poorly differentiated tumours showing both LOH at, and aberrant expression of, TP53 can be explained mechanistically. In contrast, BRCA1 mutations are rare in sporadic breast cancer, suggesting that BRCA1 is inactivated by nonmutational mechanisms (Papa et al, 1998), since epigenetic mechanisms (e.g. promoter hypermethylation), manifested as abnormal expression, have been shown to be involved in abrogating the function of certain tumour suppressor genes (such as BRCA1), which are already targeted by LOH (Dobrovic and Simpfendorfer, 1997; Jones and Baylin, 2002). Our finding that combined LOH and abnormal expression in BRCA1 was associated with a higher tumour grade therefore re-emphasises the importance of the different mechanisms that may affect expression, and this finding is of particular tumorigenic importance in sporadic cancer, as the frequency of somatic mutation in many tumour suppressor genes involved in regulating genomic stability is extremely low. In the present study, we also found that LOH was more frequent than abnormal protein expression at ATM, BRCA1 and TP53. One possible explanation for this is that expression of the remaining allele might increase to compensate for the loss of expression due to the deleted allele (Liao et al, 2003).

The observation that the only gene of the three tested found by multigenetic logistic regression analysis to be independently associated with tumour differentiation was BRCA1 (Table 2) is consistent with the tumour spectrum observed in familial cancer syndromes caused by mutation of ATM, BRCA1 or TP53, with only BRCA1 being specifically associated with familial breast cancer (Holt et al, 1996). However, the present study showed that a joint effect of abnormalities, either LOH or abnormal expression, in ATM, BRCA1 and TP53 is also an important factor associated with poor differentiation (Tables 3 and 4). This effect can be explained by known mechanisms involving interactions between BRCA1, ATM and TP53 (Xu et al, 1999; Gatei et al, 2000; Wang, 2000; Khanna and Jackson, 2001), including (i) ATM serves as the upstream sensor and, upon DSB formation, phosphorylates BRCA1 and TP53 to trigger DSB repair and cell cycle regulation and (ii) for breast cancer formation, breast epithelial cells that are genetically unstable because of defective BRCA1-associated repair also
need to undergo checkpoint inactivation (such as ATM or TP53) in order to escape checkpoint surveillance. More importantly, our demonstration that defective DSB checkpoint/repair genes in a common functional pathway may act together, leading to poor differentiation of breast cancer, is consistent with recent evidence suggesting the joint contribution of different genes in disease aetiology and cancer development. The combination of heterozygous abnormalities in different, but functionally related, genes is known to play a causal role in the pathogenesis of certain genetic syndromes (Cressman et al, 1999; Balmain, 2002). Furthermore, support for a joint carcinogenic effect comes from previous observational studies. For instance, there is a trend towards an increased risk of breast cancer in women harbouring a greater number of putative high-risk genotypes of oestrogen-metabolising genes or nonhomologous end-joining genes (Huang et al, 1999; Fu et al, 2003). Our findings provide additional support for the possibility of a joint effect of defects in different genes and emphasise the need to examine the whole tumorigenic pathway to obtain a better insight into the molecular changes involved in cancer development and progression.

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