SUPPLEMENTARY DISCUSSION

Breast cancer risk of R1699Q variant of BRCA1

In the past 10 years, many different studies have been undertaken to characterize the R1699Q variant of BRCA1 but its precise effect remains unclear. It was shown to be defective in phospho-specific binding to BACH1[1]. However, structural and biophysical studies suggest that R1699Q is not significantly different from the wild-type BRCA1[2]. This was also supported by the trypsin sensitivity assay, which showed R1699Q behaves similarly to wild type[3]. Yet, another study suggests the R1699Q variant destabilizes BRCA1 based on a similar assay[4], making the interpretation difficult. The transcriptional activation assays have also given inconsistent results, depending on the cell line used[3-6]. A study using functional and multifactorial likelihood approaches concluded that R1699Q is associated with low to moderate risk of developing the disease compared to other clearly deleterious variant[4], while another study classified the same variant as deleterious based on cancer family history[7]. The latter study also reported one family in which R1699Q did not segregate with the disease. In summary, the breast cancer risk in R1699Q mutation carriers is likely to be higher compared to the general population, but its precise risk is not known. Future large-scale epidemiological studies may provide a better assessment of the precise risk of this variant.

In this study, we have characterized the R1699Q variant using our mouse ES cell-based assay to examine its effect on BRCA1 function. We found the R1699Q variant to result in a 10-fold reduction in ES cell survival compared to cells expressing the wild-type BRCA1. This suggested that R1699Q is deleterious. This conclusion was further

supported by our *in vivo* studies showing that R1699Q fails to rescue the embryonic lethality of *Brca1-null* mice. Our previous work has demonstrated that most BRCA1 variants that result in ES cell lethality or show reduced cell survival are high-risk variants[8]. These variants also show defect in DNA repair function or cell cycle regulation. Surprisingly, the R1699Q variant had no effect on any of these functions. Instead, we uncovered a defect in ES cell differentiation, which in part was caused by the up-regulation of a miRNA, miR-155.

Epigenetic regulation of miR-155 by BRCA1

In this study, we have focused on understanding mechanistically how BRCA1 controls miRNA-155. The up-regulation of miRNA-155 in many cancers has been reported[9-12]. Several transcription factors that can activate the miR-155 promoter have been identified including AP-1, NF-kB, SMAD4, FOXP3 and HOXA9[13-15]. However, to date there has been no insight into how miRNA-155 may be regulated epigenetically. Our study not only demonstrates that miR-155 is epigenetically regulated, but also uncovers the role of BRCA1 in this control. We found marked increase in acetylation of histones H2A and H3 on the miR-155 promoter in R1699Q mutant cells. The histone acetylation and deacetylation is regulated by the various HAT/HDAC complexes and is important for chromatin organization. The association of BRCA1 with HDAC complex has been described previously[16]. It is also reported that BRCA1-mediated repression of ER-a promoter can be reversed by HDAC inhibitor, trichostatin A[17]. As we have detected an increase in acetylation of histones H2A and H3 on the miR-155 promoter in R1699Q ES cells as well as BRCA1-deficient tumor cell lines, we predicted that the interaction of

mutant BRCA1 with the HDAC complex is reduced or disrupted. Indeed, the ChIP experiment revealed an increase in binding of HDAC2 to the miR-155 promoter in the presence of wild type BRCA1 (Figure 4E). Based on the ChIP and coimmunoprecipitate results, we conclude that R1699Q BRCA1 is defective in the interaction with HDAC complex.

The mutational analysis of miR-155 promoter indicated that the putative BRCA1 binding site is critical for the epigenetic repression. Our observation that there is no or marginal association between BRCA1 and the three promoters with putative BRCA1 binding sites (*ESSRG, CCNB1* and *STAT5A*) suggests the association of BRCA1 with the miR-155 promoter is specific. Also, it suggests that the putative BRCA1 binding sites[18] may not necessarily be a good indicator of actual association with BRCA1. A genome-wide ChIP analysis for BRCA1 may provide a better understanding of the predictive value of these putative binding sites and the role of BRCA1 in epigenetic regulation of other promoters.

Does R1699Q have any dominant negative effect?

Because R1699Q BRCA1 fails to bind to HDAC2 but can associate with the miR-155 promoter, we tested the possibility that it may have a dominant negative effect. Although we did not find any *in vitro* evidence to support this, we cannot completely rule out this possibility. Lack of dominant negative effect was also supported by the *in vivo* observation that $Brca1^{ko/+};Tg^{R1699Q}$ mice did not show any overt phenotype and $Brca1^{cko/ko};Tg^{R1699Q}$ EB cells did not show miR-155 up-regulation (data not shown).

Also, tumors from *Brca1*$^{ko/+}$;*Trp53*$^{ko/+}$;*Tg*^{R1699Q} mice that showed high miR-155 had lost the WT allele of *Brca1* (Fig. 3a,b and Supplementary Figure 4).

Different levels of miR-155 expression in BRCA1-deficient cells

Interestingly, we found a 3-4 fold increase in miR-155 level in MECs from *Brca1*$^{cko/cko}$; *K14 Cre* mice and HEK 293 cells with BRCA1 knockdown, whereas the miR-155 levels in the tumors or tumor cell lines were much higher (50-150 fold) than the controls. This difference suggests the effect of additional regulatory signals or factors that may be involved in the transcriptional regulation of miR-155 promoter in addition to the BRCA1-mediated epigenetic control. And, these signals may depend upon the physiological or topological state of the cell. Such differences are also visible in the cells of the R1699Q ES cell derived embryoid bodies that are genotypically identical (Figure 2b). Human tumors samples also exhibit a similar variation in their pattern of miR-155 expression (Figure 5d). We also observed substantial differences in the increase of miR-155 expression between human and mouse tumors (3-6 fold compared to 50-180 fold). We attribute this to the difference in the quality of samples used for RNA extraction. For mouse tumors we used freshly-frozen samples whereas for human tumors we extracted RNA from 5-15 years old archived FFPE sections.

miR-155 as a biomarker

This study also shows a correlation between BRCA1 deficiency and miR-155 up-regulation in BRCA1-deficient tumors. However, because multiple transcription factors regulate the miR-155 promoter, it can be activated by other signals, unrelated to BRCA1.

Indeed, we observed high levels of miR-155 in one of the four tumors from Her2/Neu transgenic mice as well as in 10 cases of human breast tumors that were BRCA1 positive. Therefore, miR-155 alone may not be sufficient to determine the functional status of BRCA1. However, miR-155 may be part of a metagene signature that may be useful to determine the functional status of BRCA1. Future studies will be focused on evaluating the use of miR-155 expression to determine the BRCA1 status.

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