Table 1. Relative expression levels of BRCA1, NBR1(1A), NBR1(1B) and NBR2 in permanent human breast cell lines[a]

	BRCA1	***NBR1A***		***NBR1B***	***NBR2***
		Long	Short		
HBL100	100.0	100.0	100.0	100.0	100.0
MCF7	73.6	55.1	33.1	46.3	89.4
T47D	7.5	18.3	6.7	13.3	142.6
SK-BR-3	22.8	28.8	13.9	14.6	159.7
MDA-MB-231	200.8	160.0	29.7	126.6	133.7

[a] Results from three independent experiments were normalized to the *β-ACTIN* level of expression and are represented as a percentage of the expression levels of the HBL-100 control, non-malignant, cell line.

Table 2. Relative expression levels of BRCA1, NBR1(1A), NBR1(1B) and NBR2 in primary human breast cell cultures [a]

	BRCA1	***NBR1A***		***NBR1B***	***NBR2***
		Long	Short		
NME	100.0	100.0	100.0	100.0	100.0
BT15	35.7	20.2	16.6	11.4	35.8
BT18	54.5	26.8	42.3	20.6	55.0
BT19	40.3	28.8	40.3	2.1	71.5
BT27	69.9	19.9	34.1	16.9	41.0
BT101	43.0	47.6	49.2	0.0	62.7
BT117	50.4	38.8	79.0	3.9	74.2
MBC1	43.2	43.8	49.4	0.0	62.7
MBC3	56.7	0.0	75.2	0.0	82.2

[a] Results from three independent experiments were normalized to the *β-ACTIN* level of expression and are represented as a percentage from the NME (non-malignant control cell culture) expression levels.

(Nomura et al., 1994). Here, we examined the presence of the *NBR1(1A)* alternative transcript in normal and malignant epithelial breast cells. Three μg of total RNA from each of five permanent cell lines derived from normal and malignant mammary tissue, HBL-100, MCF7, T47D, SK-BR-3 and MDA-MB-231, were reverse transcribed, and the cDNA fragments were PCR amplified with gene-specific forward primers from exons 1A and 1B and reverse primers from exons 5 and 8. The resulting fragments were of the expected size and indicated that both isoforms of the *NBR1* transcript, utilizing either exon 1A or exon 1B as the first exon, were expressed in normal (HBL-100) and cancerous breast cells (Fig. 2). Moreover, exon 1A was expressed with its two variants – a short one, encompassing only 131 bp downstream of the transcription start site, and a longer one, spanning further 332 bp of the genomic sequence, as it was previously seen in human testis (Dimitrov et al., 2001). Apparently, this is the first finding of the *NBR1(1A)* splicing variant transcribed in a non-malignant somatic tissue.

Evaluation of BRCA1, NBR1 and NBR2 gene expression in permanent breast epithelial cell lines

To address whether *BRCA1* mRNA steady-state levels could be influenced by the transcription of *NBR1(1A)* and *NBR2* genes, a semi-quantitative RT-PCR was performed of the total RNA samples extracted from the five permanent cell lines. After reverse transcription, cDNA fragments of *BRCA1, NBR1(1A), NBR1(1B), NBR2, GAPDH* and *β-ACTIN* genes were amplified using gene-specific primers. The number of cycles for each amplification was predetermined to ensure linearity between the starting amount of the cDNA template and the amount of the PCR product (see Material and Methods). After quantification, the results were independently normalized to both *β-ACTIN* and *GAPDH* levels of expression. No significant variation between the two normalized data sets was observed.

The HBL-100 cell line, derived from normal mammary tissue, was used as a reference to which the expression levels of the breast cancer cell lines were compared. The relative quantification showed that three out of four breast cancer cell lines (MCF7, T47D and SK-BR-3) had decreased expression levels of *BRCA1* and all isoforms of the *NBR1* gene. The decrease was only modest in MCF7 cells but it was five- to ten-fold in T47D and SK-BR-3 cells (Fig. 3, Table 1). In contrast, the MDA-MB-231 cell line expressed both genes at higher levels than HBL-100 cells. The expression pattern of the *NBR2* gene differed from that of *NBR1*, being significantly higher in all cancer permanent cell lines, except in MCF7. No correlation between the expression levels and the oestrogen-receptor (ER) expression status of the cell lines was detected, with MCF7 and T47D known to be (ER+) and HBL-100, SK-BR-3 and MDA-MB-231 being (ER-) (Jeffy et al., 1999; Schneider et al., 2000).

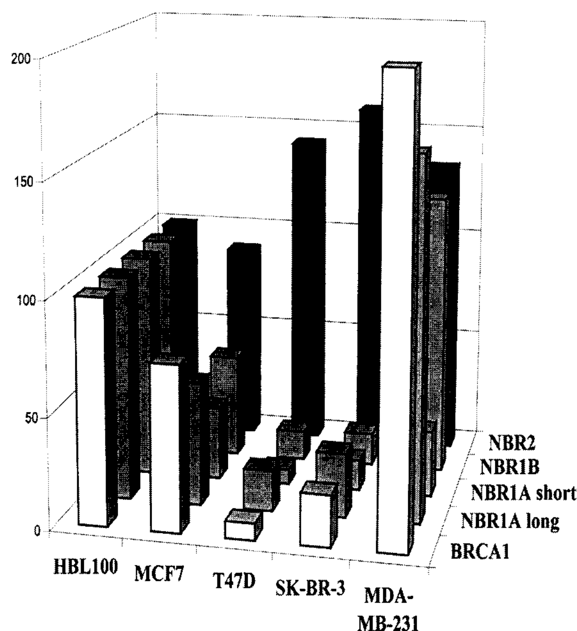

Fig. 3. Relative expression levels of *BRCA1, NBR1(1A), NBR1(1B)* and *NBR2* genes in permanent cell lines MCF7, T47D, SK-BR-3, MDA-MB-231 and HBL-100. Data were normalized to *β-ACTIN* levels of expression and are presented as a percentage of the expression levels of the corresponding genes of the normal breast-derived HBL-100 cell line.

Evaluation of BRCA1, NBR1 and NBR2 gene expression in primary breast epithelial cell cultures

Further data on the expression levels of *BRCA1, NBR1* and *NBR2* genes in breast cells were obtained by examining nine primary breast cancerous and normal cell cultures. The cells, kept at an early passage, were isolated from six breast tumours (BT15, BT18, BT19, BT27, BT101 and BT117) and two metastases (MBC1 and MBC3). NME cells were derived from normal breast tissue and served as a reference for the expression study. The results from semi-quantitative RT-PCR were consistent with the results obtained with the permanent cell lines. *BRCA1, NBR1(1A)* and *NBR1(1B)* mRNA steady-state levels showed a significant decrease in all cancer cell populations (Table 2). In contrast to permanent cell lines, the *NBR2* gene showed a lower level of expression in the primary tumour cells, though the decrease was much lower than in the case of *BRCA1*.

Discussion

The frequent inactivation of the *BRCA1* gene in the familial cases of breast cancer through germ-line mutations in the coding region and invariable loss of the wild-type allele (Welcsh and King, 2001) implied a tumour suppressor function for the gene and an important role in breast tumour formation. The significantly decreased levels of *BRCA1* mRNA and protein product in sporadic breast cancer, together with the reported frequent loss of heterozygosity (LOH) for the locus in these cases, supported a role for *BRCA1* also in the sporadic breast carcinogenesis. However, the mechanism responsible for the observed reduced expression levels of *BRCA1* in breast cancer remains largely unknown, since no mutations in the coding region of the gene or in the promoter region were found (Futreal et al., 1994; Catteau et al., 1999b) and promoter methylation was reported only in 11–13% of the sporadic cases of breast cancer (Catteau et al., 1999a; Esteller et al., 2000). An alternative explanation of the decreased mRNA levels of the gene in malignant tissue could be an increased mRNA instability or a transcriptional deregulation of *BRCA1*.

Our finding of new putative domains in the predicted protein sequence of NBR1, conserved in p62 and ref(2)P proteins, suggests that these three genes may represent a novel gene family with a role in the cellular ubiquitin system and in spermatogenesis. This observation corresponds well with the recently identified function of *BRCA1* gene product in the ubiquitination pathway and supports the hypothesis of co-ordinated expression of the genes.

The permanent breast cancer-derived cell lines represent a suitable model for the study of this phenomenon, since no aberrant *BRCA1*-promoter methylation was detected in the vast majority of them. A good example is the widely studied MCF7 cell line, which shows reduced levels of *BRCA1* mRNA (Thompson et al., 1995; Ribieras et al., 1997), yet it displays a normal methylation pattern of all examined promoter regions. All permanent cell lines used in this study were shown to be unmethylated at the *BRCA1* locus (Esteller et al., 2000).

Recently, we described the reciprocal mode of expression of *Brca1* and *Nbr1(1a)* transcripts during the mouse spermatogenesis and a high conservation between *Brca1/Nbr1(1a), BRCA1/NBR2* and *pseudoBRCA1/NBR1(1A)* bi-directional promoters (Dimitrov et al., 2001). To address the question of a possible relationship between the transcription of *BRCA1* and *NBR1/NBR2* genes in human somatic tissue, their mRNA levels were examined in a number of normal and malignant breast cell lines and primary cell cultures. We have demonstrated that *BRCA1* and *NBR1* genes are downregulated in all examined breast cancer-derived cell lines and primary cell cultures, with the exception of the MDA-MB-231 line. Moreover, our results show, for the first time, that the *NBR1(1A)* splice isoform occurs in normal somatic tissue. Until now it was detected in a human myeloblast cell line and in mouse and human spermatids (Dimitrov et al., 2001). The pattern of *NBR2* expression differed. In permanent