Sex-biased differences in the correlation between epithelial-to-mesenchymal transition-associated genes in cancer cell lines

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Abstract. There is a wide disparity in the incidence, malignancy and mortality of different types of cancer between each sex. The sex-specificity of cancer seems to be dependent on the type of cancer. Cancer incidence and mortality have been demonstrated as sex-specific in a number of different types of cancer, such as liver cancer, whereas sex-specificity is not noticeable in certain other types of cancer, including colon and lung cancer. The present study aimed to elucidate the molecular basis for sex-biased gene expression in cancer. The mRNA expression of the epithelial-to-mesenchymal transition-associated genes was investigated, including E-cadherin (also termed CDH1), vimentin (VIM), discoidin domain receptor 1 (DDR1) and zinc finger E-box binding homeobox 1 (ZEB1) in female- and male-derived cancer cell lines by reverse transcription (RT)-PCR and the Broad-Novartis Cancer Cell Line Encyclopedia (CCLE) database analysis. A negative correlation was observed between DDR1 and ZEB1 only in the female-derived cancer cell lines via RT-PCR analysis. A negative correlation between DDR1 index (defined by the logarithmic value of DDR1 divided by ZEB1, based on the mRNA data from the RT-PCR analysis) and an invasive phenotype was observed in cancer cell lines in a sex-specific manner. Analysis of the CCLE database demonstrated that DDR1 and ZEB1, which are already known to be sex-biased, were negatively correlated in female-derived liver cancer cell lines, but not in male-derived liver cancer cell lines. In contrast, cell lines of colon and lung cancer did not reveal any sex-dependent difference in the correlation between DDR1 and ZEB1. Kaplan-Meier survival curves using the transcriptomic datasets such as Gene Expression Omnibus, European Genome-phenome Archiva and The Cancer Genome Atlas databases suggested a sex-biased difference in the correlation between DDR1 expression pattern and overall survival in patients with liver cancer. The results of the present study indicate that sex factors may affect the regulation of gene expression, contributing to the sex-biased progression of the different types of cancer, particularly liver cancer. Overall, these findings suggest that analyses of the correlation between DDR1 and ZEB1 may prove useful when investigating sex-biased cancers.

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Introduction

There is mounting evidence to support the sex-biased differences in the susceptibility, incidence and mortality of cancer (1-3). The incidence of cancer and the mortality rates were higher in men than in women in the USA between 2008 and 2014 (4). The sex-specificity of cancer seems to be dependent on the type of cancer. In certain types of cancer, such as melanoma, esophagus, larynx, liver and bladder cancer, the incidence and mortality have been demonstrated to be sex-specific (4-6). In particular, there are prominent differences between men and women in liver cancer, with almost three times higher incidence rate in men than in women, according to the data reported in 2018 (4,7-9). On the other hand, in colorectal and lung cancer, which are the most commonly diagnosed malignancies and the most common leading cause of cancer-associated mortality (7,10,11), remarkable sex-specificity has not been reported.

In addition to lifestyle and living environments, genetic factors also play an essential role in the wide disparity between the sexes in terms of cancer incidence and mortality of cancer (2,3,12-14). The expression of cancer-associated genes was correlated with sex differences in cancer and thus, these genes were designated as sex-affected (3,12,14). However, to the best of our knowledge, there is limited evidence supporting sex differences in the correlation of gene expression.

Cancer cell lines have been used to investigate the underlying mechanisms, prevention, diagnosis and treatment of

cancer. The sex description of cell lines, however, is often inadequate (15). The majority of the animal models used for verification of *in vitro* results primarily use male animal models, as the changes in hormone levels associated with the female menstrual cycle can affect the experimental results (16-18).

The epithelial-to-mesenchymal transition (EMT), a cellular process involving changes in cell shape, adhesion and movement, has been associated with cancer progression and metastasis (19-21). The transdifferentiation of epithelial cells into mesenchymal cells requires a number of molecular changes, including the repression of E-cadherin, an epithelial phenotype-associated protein (22-26). Transcription factors, such as Snail 1 (SNAI1), Slug (SNAI2), Zinc finger E-box binding homeobox (ZEB)1 and ZEB2, were revealed to bind and repress the promoter region of the E-cadherin gene CDH1 (21). Vimentin (VIM), a major constituent protein of the intermediate filament family, is known to be overexpressed during the EMT process (27,28).

Discoidin domain receptors (DDRs) are receptor tyrosine kinases that are activated by collagen (29). The roles of DDR1 in the EMT have been reported in various types of cancer (30). DDR1 improved the stability of E-cadherin via formation of the DDR1/E-cadherin complex (31) and triggered epithelial cell differentiation by promoting cell adhesion through stabilizing E-cadherin (32). Loss of DDR1 was observed during the EMT in epithelial ovarian cancer (33). In contrast, DDR1 has been revealed to induce the EMT in renal cancer cells (34). Previously, it has been demonstrated that DDR1 was repressed by ZEB1 in breast epithelial cells undergoing H-Ras-induced EMT (35).

In the present study, the sex differences in the expression of EMT-associated genes CDH1, VIM, DDR1 and ZEB1 were investigated in various cancer cell lines. Furthermore, the gender specificity of the correlation between the expression of EMT-associated genes was also examined.

Materials and methods

Cell culture. MCF7, MDA-MB-231 and Hs578T human breast cancer cell lines were purchased from the Korean Cell Line Bank (KCLB; Korean Cell Line Research Foundation) and cultured as previously described (36). PC-3 and DU-145 human prostate cancer cell lines, and SNU-840 and SK-OV-3 human ovarian cancer cell lines were purchased from the KCLB; Korean Cell Line Research Foundation and cultured in Roswell Park Memorial Institute (RPMI) medium with L-glutamine and 25 mM HEPES, supplemented with 10% fetal bovine serum (Corning Inc.) and 100 μg/ml penicillin-streptomycin. SNU-387, SNU-449 and SNU-878 human hepatocellular carcinoma cell lines, SK-Hep1 human hepatic adenocarcinoma cell line and HepG2 human hepatoblastoma cell line were provided by Dr Sang Geon Kim (Seoul National University). The cells used in this study were maintained in a humidified atmosphere with 95% air and 5% CO_2 at 37˚C.

Comparison of mRNA expression presented in public databases. The mRNA expression levels of CDH1, VIM, DDR1 and ZEB1 in human cancer cell lines derived from the Broad-Novartis Cancer Cell Line Encyclopedia (CCLE) database (portals.broadinstitute.org/ccle) were compared in the present study. The sex origin of each cell line was confirmed at the American Type Culture Collection website (https://www.atcc.org) and SciCrunch (https: //www.scicrunch.org). The correlation between CDH1, VIM, DDR1 and ZEB1 was analyzed using Statistical Analysis System (SAS 9.1.3; SAS Institute, Inc.) and represented as Pearson correlation coefficients (r) and P-values to measure the significance.

Reverse transcription-quantitative (RT)-PCR. Total RNA was extracted from male- and female-derived cancer cells using TRIsure™ (Bioline), according to the manufacturer's protocol. The RNA was reverse-transcribed to obtain cDNA using the Tetro Reverse Transcriptase cDNA Synthesis kit (Bioline) according to the manufacturer's protocol. The PCR cycling conditions and primer sequences for CDH1, VIM, DDR1, ZEB1 and β-actin were performed as previously described (35,37). The primer sequences were as follows: Human CDH1: Forward, 5'-TCCATTTCTTGG TCTACGCC-3', reverse, 5'-CACCTTCAGCCATCCTGTTT-3'; VIM: Forward, 5'-GGCTCAGATTCAGGAACAGC-3', reverse, 5'-GTCTCAACGGCAAAGTTCTC-3'; human DDR1: Forward, 5'-GGACATACCGTGGGCGGACT-3', reverse, 5'-CCTAGG TTGTGGCGCATGG-3'; human ZEB1: Forward, 5'-GCACAA CCAAGTGCAGAAGA-3', reverse, 5'-GAACCATTGGTGGTT GATCC-3'; and human β-actin: Forward, 5'-ACTCTTCCAGCC TTCCTT-3', reverse, 5'-TCTCCTTCTGCATCCTGTC-3'. The following amplification conditions were applied for the PCR of human ZEB1 and CDH1: 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 1 min for 27 cycles; human VIM, 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 45 sec for 28 cycles; and human DDR1, 94˚C for 30 sec, 57˚C for 30 sec, and 72˚C for 1 min for 28 cycles. PCR product (10 μl) synthesized using the MyTaq™ kit (Bioline) were analyzed by electrophoresis using gel with 1% agarose and 0.1 μg/ml ethidium bromide (EtBr) and bands of DDR1 (320 bp), ZEB1 (284 bp), CDH1 (361 bp), VIM (327 bp) and β-actin (175 bp) were detected and quantified using Image Lab™ Software (version 5.2; Bio-Rad Laboratories, Inc.).

Immunoblot analysis. Immunoblot analysis was performed as previously described (38). The antibodies used in the present study included polyclonal rabbit anti-ZEB1 (cat. no. sc-25388; 1:1,000), polyclonal rabbit anti-CDH1 (cat. no. sc-7870; 1:1,000), polyclonal rabbit anti-DDR1 (cat. no. sc-532; 1:1,000) primary antibodies obtained from Santa Cruz Biotechnology, Inc. The monoclonal rabbit anti-VIM primary antibody (cat. no. 5741S; 1:1,000) were obtained from Cell Signaling Technology, Inc. The monoclonal mouse anti-β-actin primary antibody (cat. no. A2228; 1:4,000; Sigma-Aldrich; Merck KGaA) was also used. Goat anti-rabbit (cat. no. 65-6120; 1:4,000) and goat anti-mouse (cat. no. 62-6520; 1:4,000) were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Individual membranes were incubated with appropriately diluted primary antibodies (1:1,000) overnight at 4˚C. Horseradish peroxidase (HRP)-conjugated secondary antibodies (1:4,000) were then applied for 1.5 h at room temperature, following three intensive washes in phosphate buffered saline with Tween 20 (PBST). The protein expression levels were examined using enhanced chemiluminescence (WesternBright™ ECL; Advansta Inc, Menlo Park, CA, USA) and detected using the Gel Doc™ XR+ system (Bio-Rad Laboratories, Inc.). Relative band intensities were determined by the quantification of each band using Image Lab software (version 5.2; Bio-Rad Laboratories, Inc.).

Table I. Relative correlation of mRNA expression between EMT-associated genes and ZEB1.

Sex origin of cell lines	Organ	Correlation	Correlation coefficients (P-value)		
			ZEB1 vs. Log CDH1	ZEB1 vs. Log VIM	ZEB1 vs. Log DDR1
Female	Breast	Pearson (r)	-0.8154 (P<0.0010)	0.6947 (P<0.0010)	-0.8210 (P<0.0010)
		Spearman (ρ)	-0.5738 (P<0.0010)	0.6144 (P<0.0010)	-0.6072 (P<0.0010)
	Ovary	Pearson (r)	-0.7944 (P<0.0010)	0.5823 (P<0.0010)	-0.7324 (P<0.0010)
		Spearman (ρ)	-0.7743 (P<0.0010)	0.6772 (P<0.0010)	-0.7703 (P<0.0010)
	Liver	Pearson (r)	-0.9101 (P=0.2720)	-0.7579 (P=0.4524)	-0.8043 (P=0.4051)
		Spearman (ρ)	-0.5000 (P>0.9999)	-1.000 (P=0.3333)	-1.000 (P=0.3333)
	Colon	Pearson (r)	-0.8267 (P<0.0010)	0.8074 (P<0.0010)	-0.6324 (P<0.0100)
		Spearman (ρ)	-0.3168 (P=0.2002)	0.6367 (P<0.0100)	-0.3829 (P=0.1168)
	Lung	Pearson (r)	-0.8152 (P<0.0010)	0.5817 (P<0.0010)	-0.7604 (P<0.0010)
		Spearman (ρ)	-0.7997 (P<0.0010)	0.6715 (P<0.0010)	-0.8132 (P<0.0010)
Male	Prostate	Pearson (r)	-0.7579 (P<0.0500)	0.9089 (P<0.0100)	0.1431 (P=0.7352)
		Spearman (ρ)	-0.8095 (P<0.0500)	0.8571 (P<0.0500)	0 (P>0.9999)
	Liver	Pearson (r)	-0.7583 (P<0.0010)	0.4025 (P=0.0569)	0.2164 (P=0.3214)
		Spearman (ρ)	-0.5741 (P<0.0100)	0.5474 (P<0.0100)	0.1848 (P=0.3986)
	Colon	Pearson (r)	-0.8898 (P<0.0010)	0.7627 (P<0.0010)	-0.6699 (P<0.0010)
		Spearman (ρ)	-0.5444 (P<0.0100)	0.3020 (P=0.0987)	-0.1887 (P=0.3093)
	Lung	Pearson (r)	-0.7730 (P<0.0010)	0.4784 (P<0.0010)	-0.5250 (P<0.0010)
		Spearman (ρ)	-0.7589 (P<0.0010)	0.5000 (P<0.0010)	-0.5359 (P<0.0010)

EMT, epithelial-to-mesenchymal transition; ZEB1, zinc finger E-box binding homeobox 1; VIM, vimentin; DDR1, discoidin domain receptor 1; NS, not significant.

In vitro transwell invasion assay. *In vitro* transwell invasion assays were performed as previously described (39). Using a 24-well transwell insert with membranes of 8.0-μm pores (Falcon; Corning Inc.), the lower part of the membrane was covered with 0.5 mg/ml type I collagen (Corning Inc.), and the upper part was covered in reconstituted basement membrane material, 0.5 mg/ml matrigel (BD Biosciences) and then dried. Complete medium (600 μl) was inserted into the bottom of the well and 100 μl serum-free medium containing $5x10^4$ cells was placed in the upper chamber of the transwell. Cells were incubated for 24 h in a humidified atmosphere with 5% CO_2 at 37˚C. Following incubation, the invaded cells attached to the membrane were stained by 0.5% crystal violet for 20 min at room temperature. For cell imaging, the filter membrane was cut and fixed with Canada balsam (Junsei Chemical Co., Ltd.) on the glass slide. The invaded cells in the lower side of the filter were viewed under the by microscope (Olympus CK2) at x100 magnification and the images were captured with the camera attached to a microscope (Olympus U-PMTVC). For quantitative measurements, the membrane filter stained with crystal violet was cut out and eluted with 30% methanol for 5 min. The absorbance was measured at a wavelength of 595 nm.

In order to illustrate the correlation between the invasiveness of each cell line and mRNA expression of EMT-associated molecules, the levels of mRNA expression of EMT-associated molecules were converted into logarithmic values. The DDR1 index was defined as the logarithmic value of DDR1 divided by ZEB1, based on the mRNA data of ZEB1, CDH1, VIM and DDR1 obtained from the RT-qPCR analysis. Similarly, CDH1 index and VIM index were defined as the logarithmic value of CDH1 divided by ZEB1, and that of VIM divided by ZEB1, respectively.

Kaplan-Meier survival curves. Analyses including the Kaplan-Meier survival curves were calculated and plotted by The Kaplan Meier Plotter (http://kmplot.com) using the transcriptomic datasets, the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/), the European Genome-phenome Archive (EGA; https://ega-archive.org/) and The Cancer Genome Atlas (TCGA; version 2016_01_28; https://cancergenome.nih.gov/) repositories (40,41). The data of female and male patients with liver cancer were split using the auto select best cutoff criteria. Median mRNA expression levels of DDR1 (female, 16.3903; male, 15.9004) and ZEB1 (female, 15.9808; male, 15.9159) were used to differentiate between high expression and low expression).

Statistical analysis. The correlations of gene expression were analyzed using the Pearson and Spearman correlation with GraphPad Prism (version 6.0; GraphPad Software, Inc.). Its R^2 value (the coefficient of determination), r value (Pearson correlation coefficient), ρ value (Spearman correlation coefficient) and the significance (two-tailed P-value) of each correlation analysis are represented in Figures and listed in Table I. The significance of differences in the invasive ability of cancer cell lines from *in vitro* transwell invasion assay was analyzed using one-way analysis of variance and with Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

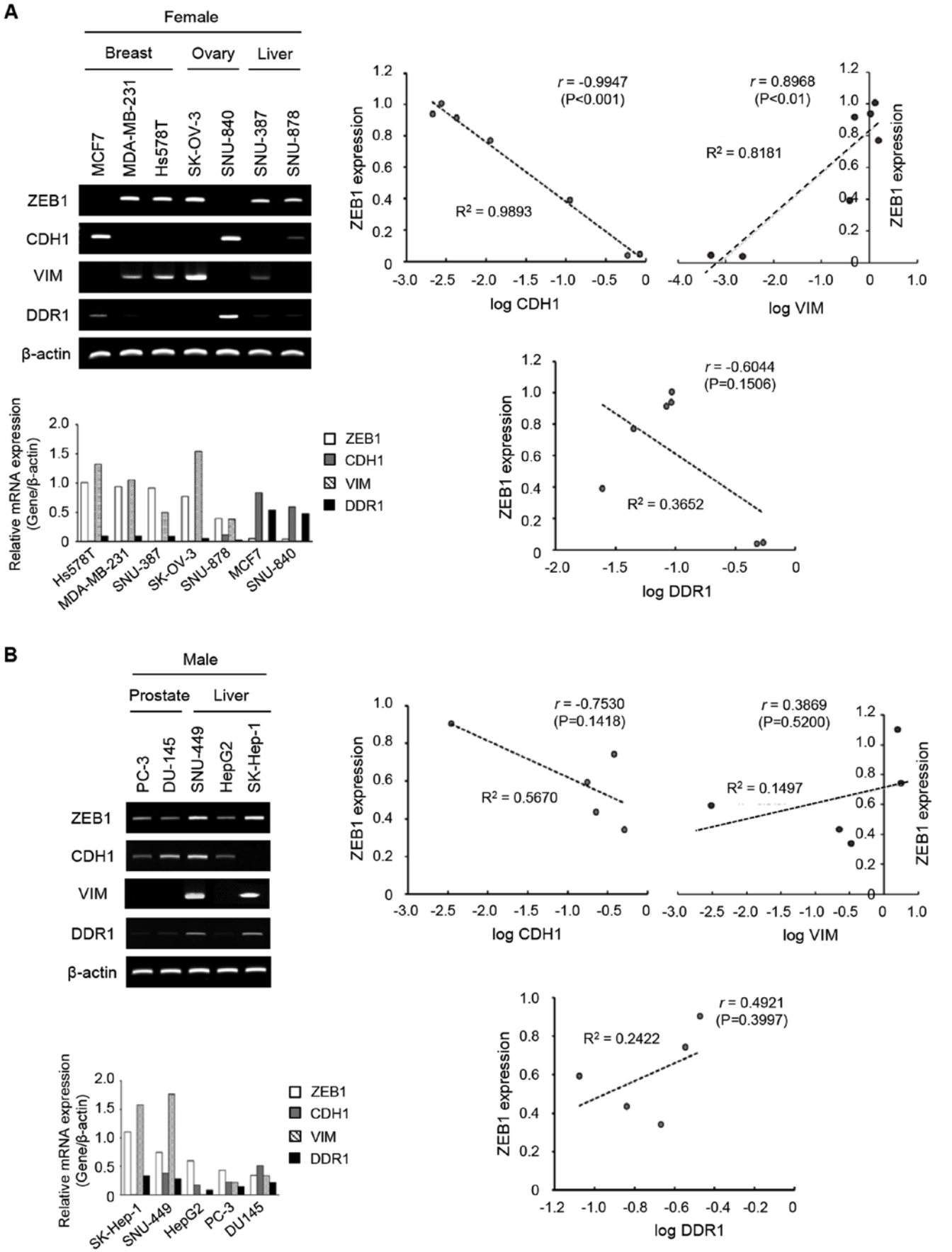

Figure 1. mRNA and protein levels of ZEB1, CDH1, VIM and DDR1 were detected by reverse transcription-PCR and immunoblot analyses in cancer cell lines. Left-hand sides, the bands of ZEB1, CDH1, VIM and DDR1 were quantified as relative values to β-actin as a loading control. Right-hand sides, the level of ZEB1 was plotted against the logarithmic values of CDH1, VIM or DDR1. Each graph represents a trend line with its R^2 value (the coefficient of determination) and r value (Pearson correlation coefficient). The levels of mRNA expression in the female- (A) and male- (B) derived cancer cell lines.

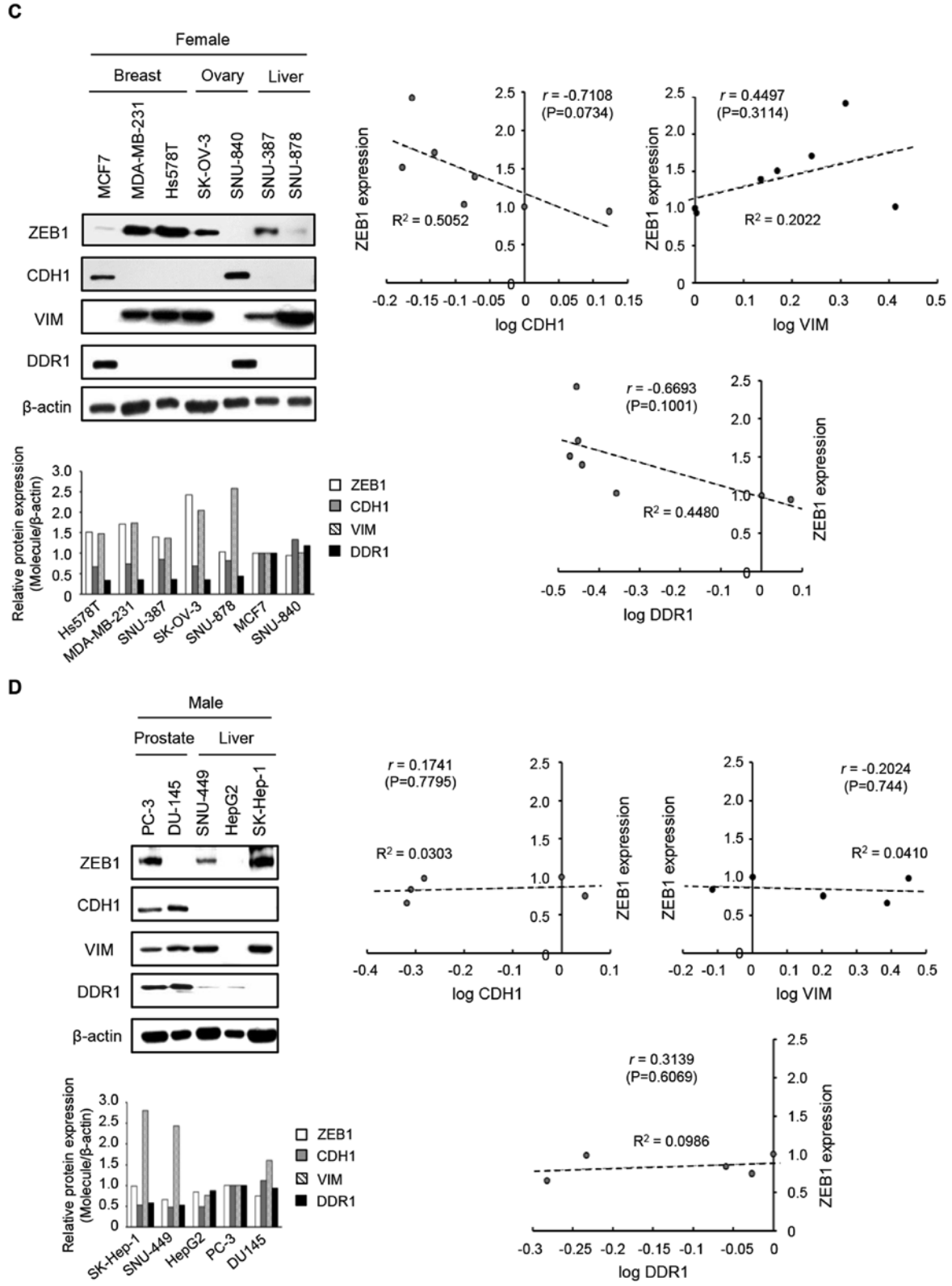

Figure 1. Continued. The levels of protein expression in the female- (C) and male- (D) derived cancer cell lines. ZEB1, zing finger E-box binding homeobox; VIM, vimentin; DDR1, discoidin domain receptor 1.

Results

Negative correlation between DDR1 and ZEB1 is observed only in female-derived cancer cell lines by RT-PCR and immunoblot analysis. In order to investigate the sex differences in the expression and correlation of EMT-associated molecules, the mRNA expression levels of CDH1, VIM, DDR1 and ZEB1 in human cancer cell lines were detected (Fig. 1). RT-PCR was performed in 7 female-derived cancer cell lines (MCF7, Hs578T, MDA-MB-231, SK-OV-3, SNU840, SNU-387 and

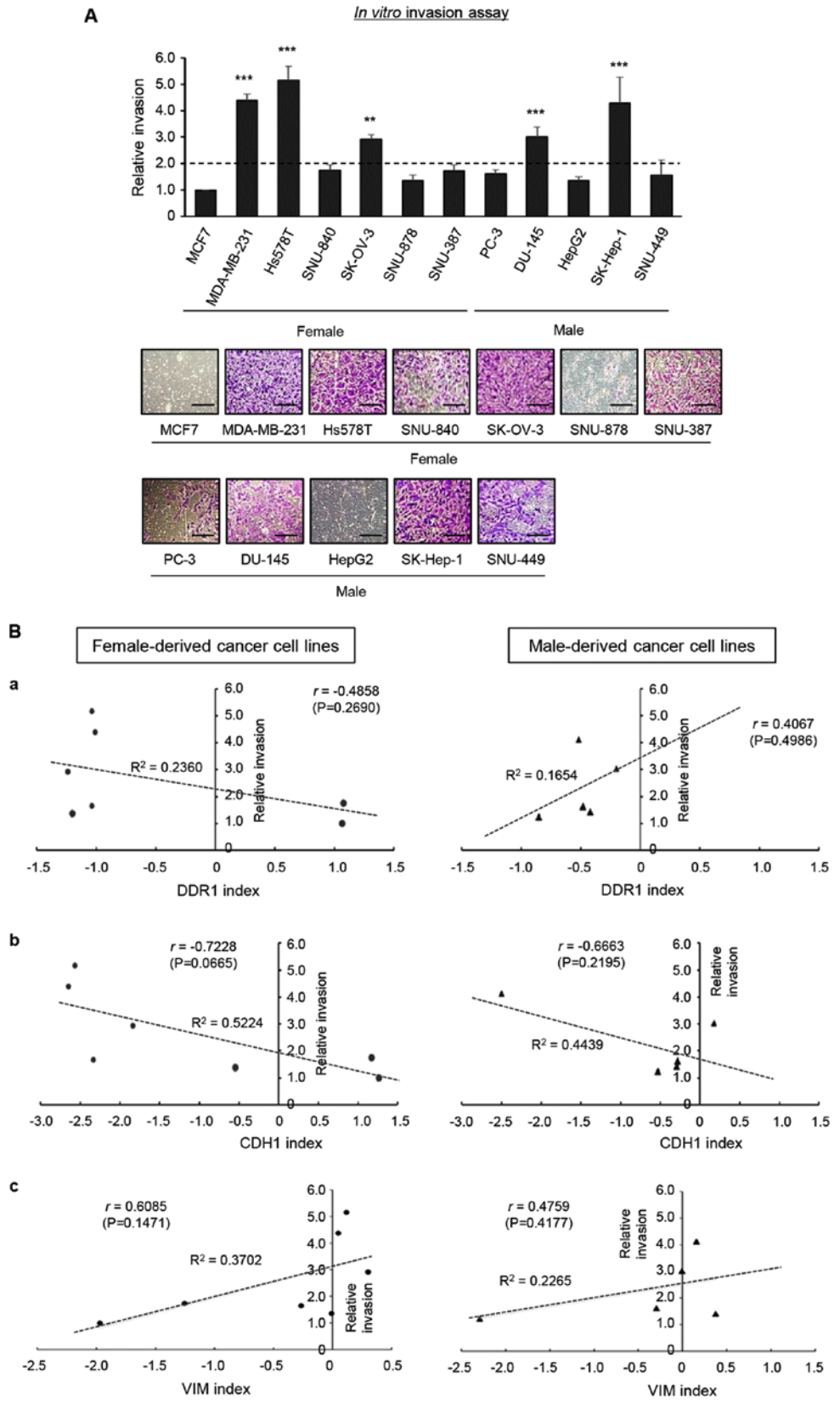

Figure 2. *In vitro* invasion assay of 7 female-derived and 5 male-derived cancer cell lines. (A) The invasive phenotypes are demonstrated by the images of the cells. (B) Relative invasion values were obtained using the invasiveness of the MCF7 cell line as a reference value. Relative invasion values were plotted against the (a) DDR1 index, (b) CDH1 index or (c) the VIM index. The index values were defined as the logarithmic values of DDR1, CDH1 or VIM divided by ZEB1. Magnification, x100. Scale bar, 100 μm. All results are presented as the mean ± standard deviation of three independent experiments performed in triplicate. **P<0.01 and ***P<0.001 vs. MCF7.

SNU-878; Fig. 1A) and 5 male-derived cancer cell lines (PC-3, DU-145, SNU-449, HepG2 and SK-Hep1; Fig. 1B). In order to observe not only the correlations between gene expression, but also the correlations between protein expression, immunoblot

analyses were conducted using the same, aforementioned cell line samples (Fig. 1C and D).

These data were statistically analyzed using the Pearson correlation coefficients of log CDH1, log VIM, or log DDR1 with ZEB1 and a curve was plotted. As presented in Fig. 1A, the plot of log CDH1 and ZEB1 revealed a negative linear correlation (r, -0.9947; P<0.001) and log VIM and ZEB1 revealed a positive linear correlation (r, 0.8968; P<0.010) in female-derived cell lines. A negative correlation was observed between DDR1 and ZEB1 (r, -0.6044; P=0.1506) in female-derived cell lines. In Fig. 1C, the correlation of protein expression of the EMT-associated molecules revealed a similar pattern to that of mRNA expressions. Expression of the same molecule was examined at the mRNA and protein levels, with similar patterns but not significantly consistent (Fig. 1). This can be explained by the results of previous studies that the expression levels of genes and proteins are not always consistent because of cellular modification following gene expression (42).

As presented in Fig. 1B, male-derived cell lines, showed a tendency towards negative correlation between log CDH1 and ZEB1 (r, -0.7530; P=0.1418), and a tendency towards positive correlation between log VIM and ZEB1 (r, 0.3869 P=0.5200). The data demonstrated a trend of negative correlation between CDH1 and DDR1 and a positive correlation between VIM and DDR1 in the female- and male-derived cell lines (Fig. 1A and B). Unlike female-derived cell lines, the correlation between log DDR1 and ZEB1 was positive in male-derived cell lines (r, 0.4921; P=0.3997; Fig. 1B). In Fig. 1D, the correlation coefficients between EMT-associated molecules and ZEB1 in male-derived cancer cell lines was lower, which suggested that the correlation between the two protein expression levels more decreased in male-derived cancer cell lines than in female-derived cancer cell lines.

These data demonstrate that the correlation between DDR1 and ZEB1 revealed a negative tendency specific to female-derived cancer cell lines, although this was not statistically significant.

Negative correlation between DDR1 index and invasive phenotype is observed in cancer cell lines in a sex-specific manner. The differences in gene expression in regard to their association with the invasive phenotype of cancer cell lines was investigated in the present study. To this end, an *in vitro* transwell invasion assay was performed and the invasive ability from three independent experiments was quantified relative to the invasiveness of MCF7 cells (Fig. 2A). Since the MCF7 cell line is widely known to be non-invasive (43), the invasiveness of each cell line was compared to this cell line. The DDR1, CDH1 and VIM index was defined as the logarithmic value of each molecule divided by ZEB1, based on the mRNA data of ZEB1, CDH1, VIM and DDR1 obtained from the RT-PCR analysis, respectively (Fig. 1A and B). A plot of the relative invasion vs. DDR1 index revealed a negative correlation only in female-derived cell lines, and not in male-derived cell lines (Fig. 2B-a). A plot of the relative invasion vs. CDH1 index or VIM index revealed that the relative invasion was negatively correlated with CDH1 index and positively correlated with VIM index in both female- and male-derived cancer cell lines (Fig. 2B-b and B-c, respectively). Although the results were not of statistical significance, due to the limited number of samples, these results suggest that DDR1 index may have the potential to serve as an indicator of invasive phenotype of female-derived cancer cell lines. However, as there were no sex-specific differences observed in the correlations between invasion and CDH1 and VIM indices, these would not be suitable as invasive phenotype indicators.

Analysis of CCLE database reveals a sex-biased difference in the correlation between DDR1 and ZEB1 in liver-derived cell lines. In order to expand the experimental results that demonstrated a negative correlation of sex difference between DDR1 and ZEB1, the Broad-Novartis CCLE database was analyzed. As the expression levels of DDR1 and ZEB1 in cancer cell lines were not sex-biased, the correlation between the expression levels of DDR1 and ZEB1 was investigated. As presented in Fig. 3A, the gene expression profiles from the CCLE database revealed that the mRNA levels of ZEB1 were negatively correlated with DDR1 with a clear, negative linear correlation in female-derived cancer cell lines. The results were as follows: 58 breast (r, -0.8210; P<0.001), 52 ovary (r, -0.7324; P<0.001), 3 female liver (r, -0.8043; P=0.4051), 18 female colon (r, -0.6324; P<0.01) and 48 female lung cancer cell lines (r, -0.7604; P<0.001).

In male-derived cancer cell lines (8 prostate, 23 male liver, 31 male colon and 119 male lung cancer cell lines), however, the negative correlation between ZEB1 and DDR1 was observed only in male colon-derived (r, -0.6699; P<0.001) and male lung-derived cell lines (r, -0.5250; P<0.001) (Fig. 3B). There was a positive correlation between ZEB1 and DDR1 in male prostate- and liver-derived cell lines (r, 0.1431; r, 0.2163, respectively) but these values were not significant as follows: Prostate (r, 0.1431; P=0.7352) and male liver (r, 0.2164; P=0.3214). Among the analyzed cancer types between the two sexes (liver, colon and lung cancers), only liver-derived cell lines revealed a sex-biased difference between DDR1 and ZEB1. Given that the marked difference between men and women were observed in liver cancer, but not in colon and lung cancers, the results of this analysis suggest that the correlation between DDR1 and ZEB1 may be used for investigating the types of cancer that are sex-biased.

Both female- and male-derived cell lines from CCLE database present a negative correlation between CDH1 and ZEB1, and a positive correlation between VIM and ZEB1. In female-derived cancer cell lines, the mRNA levels of ZEB1 were negatively correlated with CDH1 (Fig. 4A-a, A-c, A-e, A-g and A-i). The plot of log CDH1 and ZEB1 in these cell lines revealed a negative linear correlation (Fig. 4A-b, A-d, A-f, A-h and A-j). The r values of log CDH1 with ZEB1 in female breast-, ovary-, liver-, colon- and lung-derived cell lines were -0.8154 (P<0.001), -0.7944 (P<0.001), -0.9101 (P=0.2720), -0.8267 (P<0.001) and -0.8152 (P<0.001), respectively. The mRNA levels of ZEB1 in male-derived cancer cell lines were also negatively correlated with CDH1 (Fig. 4B-a, B-c, B-e and B-g). The plot of log CDH1 and ZEB1 in these cell lines revealed a negative linear association (Fig. 4B-b, B-d, B-f and B-h). The r values of the male prostate-, liver-, colon- and lung-derived cell lines were -0.7579 (P<0.05), -0.7583 (P<0.001), -0.8898 (P<0.001) and -0.7730 (P<0.001), respectively.

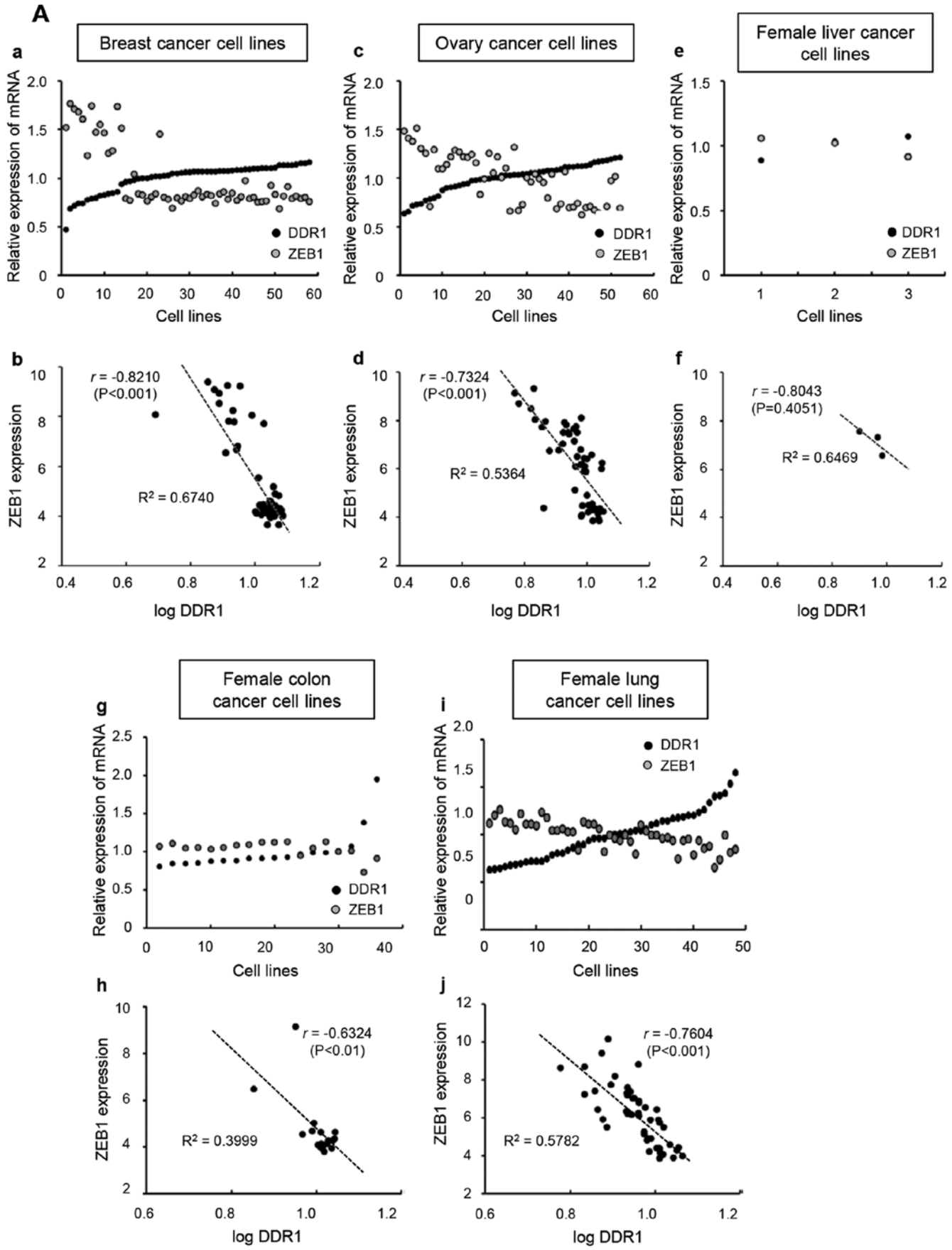

Figure 3. Broad-Novartis Cancer Cell Line Encyclopedia database analysis for mRNA levels of DDR1 and ZEB1 in cancer cell lines. Each graph presents a trend line with its R^2 value (the coefficient of determination) and r value (Pearson correlation coefficient). DDR1, discoidin domain receptor; ZEB1, zinc finger E-box binding homeobox. (A) Female-derived cancer cell lines. (a, c, e, g and i) mRNA value of DDR1 or ZEB1 in each cell line was divided by the average value of mRNA expression and plotted. Lower plots (b, d, f, h and j), ZEB1 expression levels were plotted against the logarithmic values of DDR1 levels. DDR1, discoidin domain receptor; ZEB1, zinc finger E-box binding homeobox.

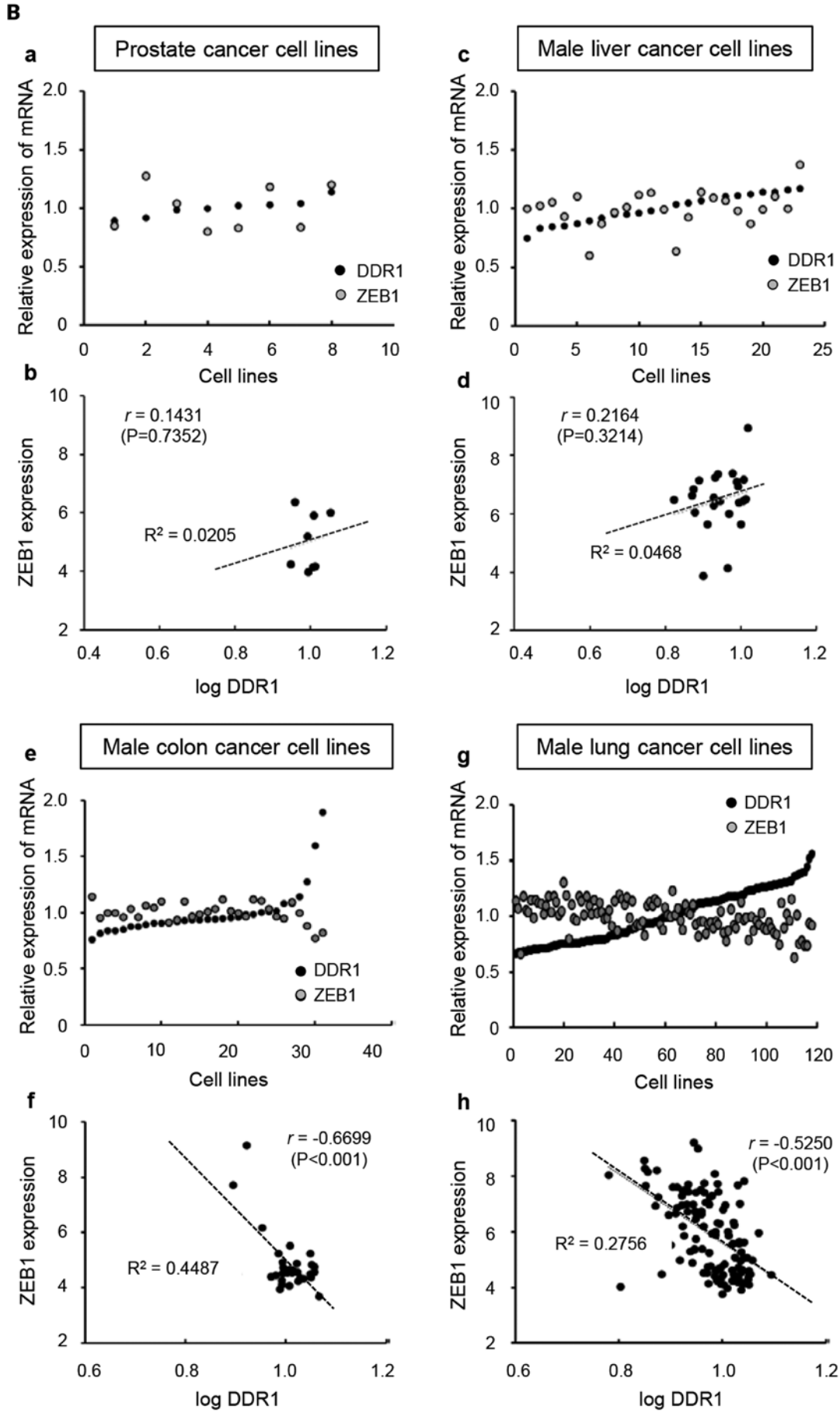

Figure 3. Continued. (B) Male-derived cancer cell lines. (a, c, e and g) mRNA value of DDR1 or ZEB1 in each cell line was divided by the average value of mRNA expression and plotted. Lower plots (b, d, f and h), ZEB1 expression levels were plotted against the logarithmic values of DDR1 levels. . DDR1, discoidin domain receptor; ZEB1, zinc finger E-box binding homeobox.

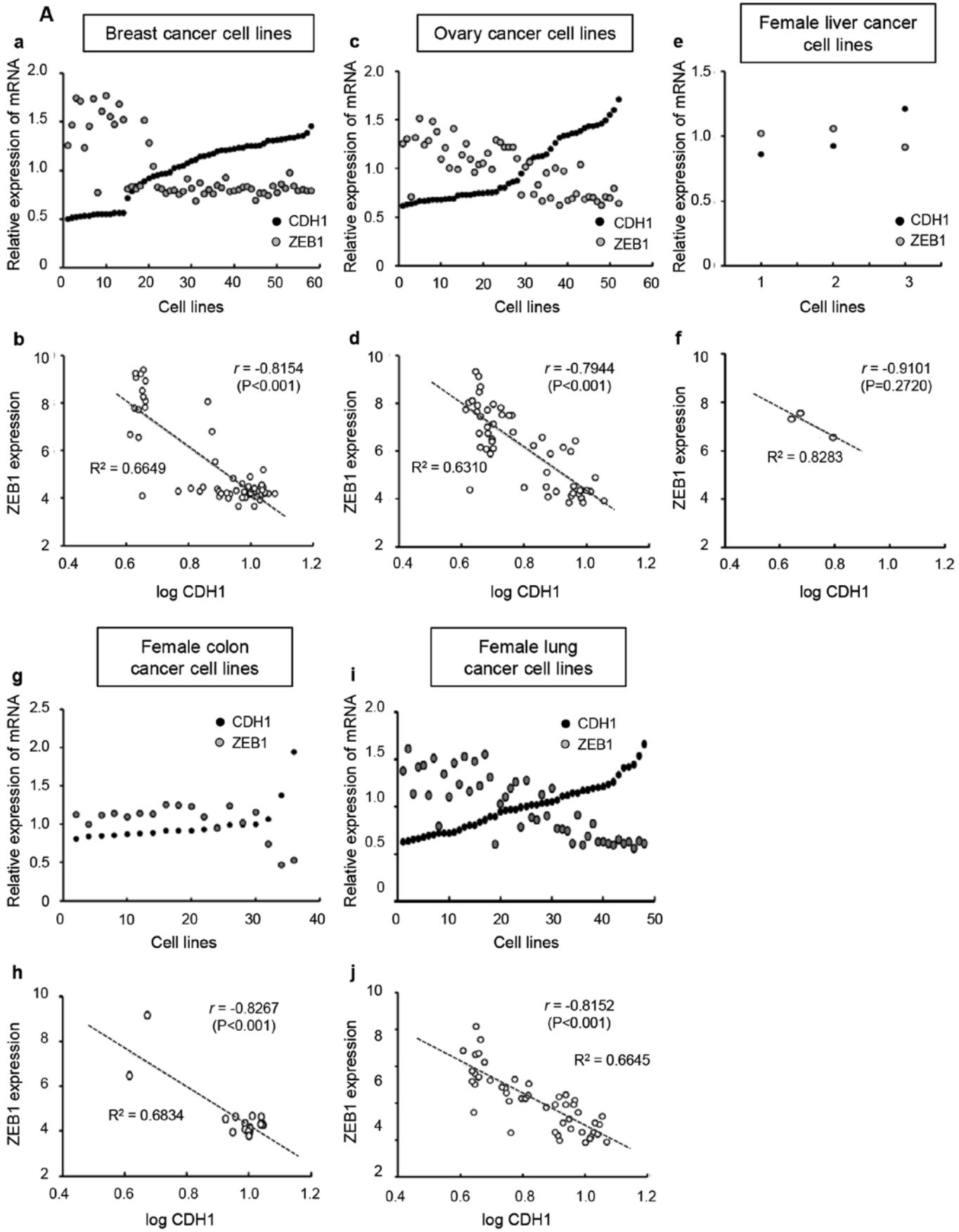

Figure 4.Broad-Novartis Cancer Cell Line Encyclopedia database analysis for mRNA levels of CDH1 and ZEB1 in cancer cell lines. Each graph presents a trend line with its R2 value (the coefficient of determination) and r value (Pearson correlation coefficient). (A) Female-derived cancer cell lines. (a, c, e, g and i) mRNA value of CDH1 or ZEB1 in each cell line was divided by the average value of mRNA expression and plotted. Lower plots (b, d, f, h and j), ZEB1 expression levels were plotted against the logarithmic values of CDH1 levels. CDH1, E-cadherin; ZEB1, zinc finger E-box binding homeobox.

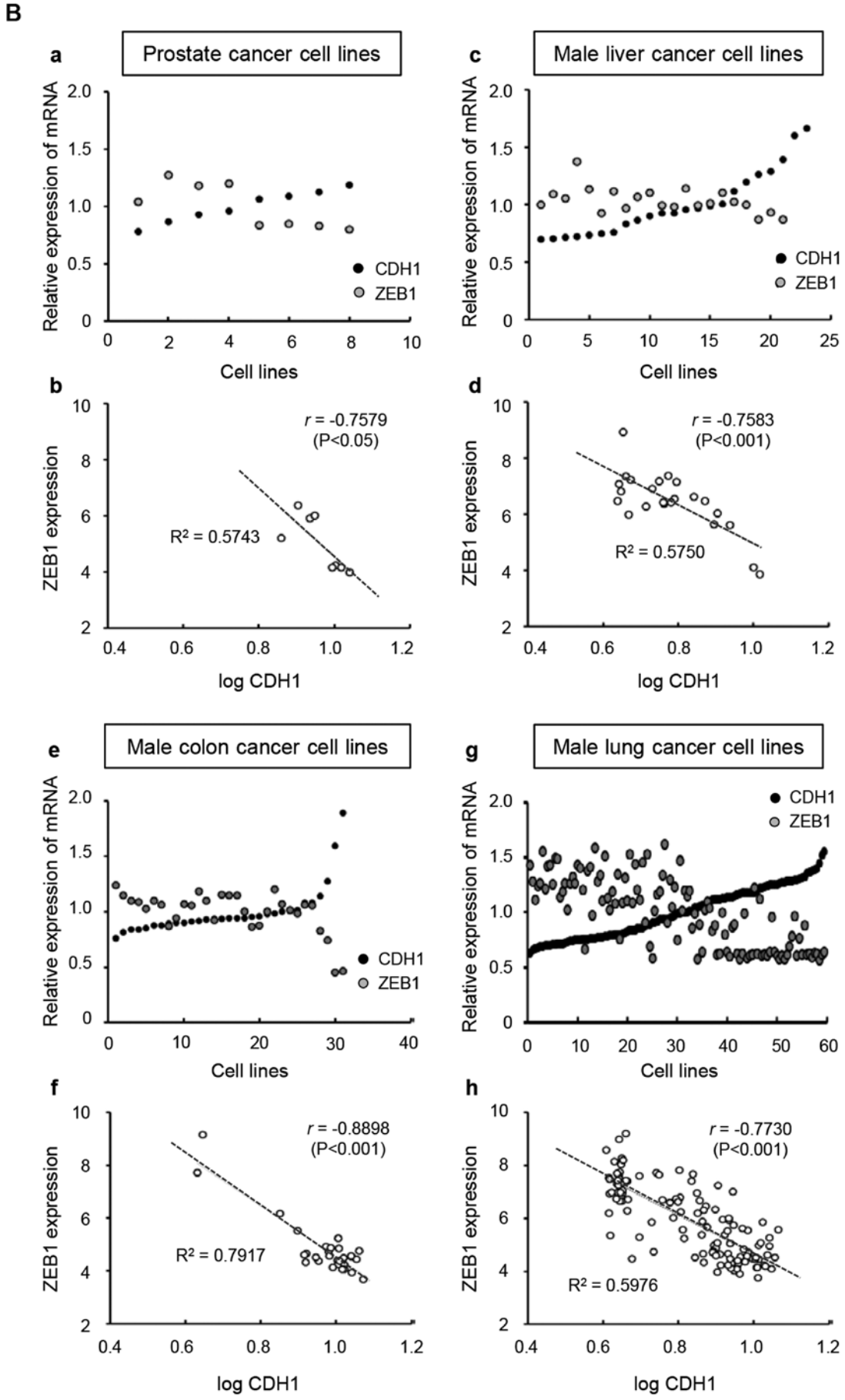

Figure 4. Continued. (B) Male-derived cancer cell lines. (a, c, e and g) mRNA value of CDH1 or ZEB1 in each cell line was divided by the average value of mRNA expression and plotted. Lower plots (b, d, f and h), ZEB1 expression levels were plotted against the logarithmic values of CDH1 levels.

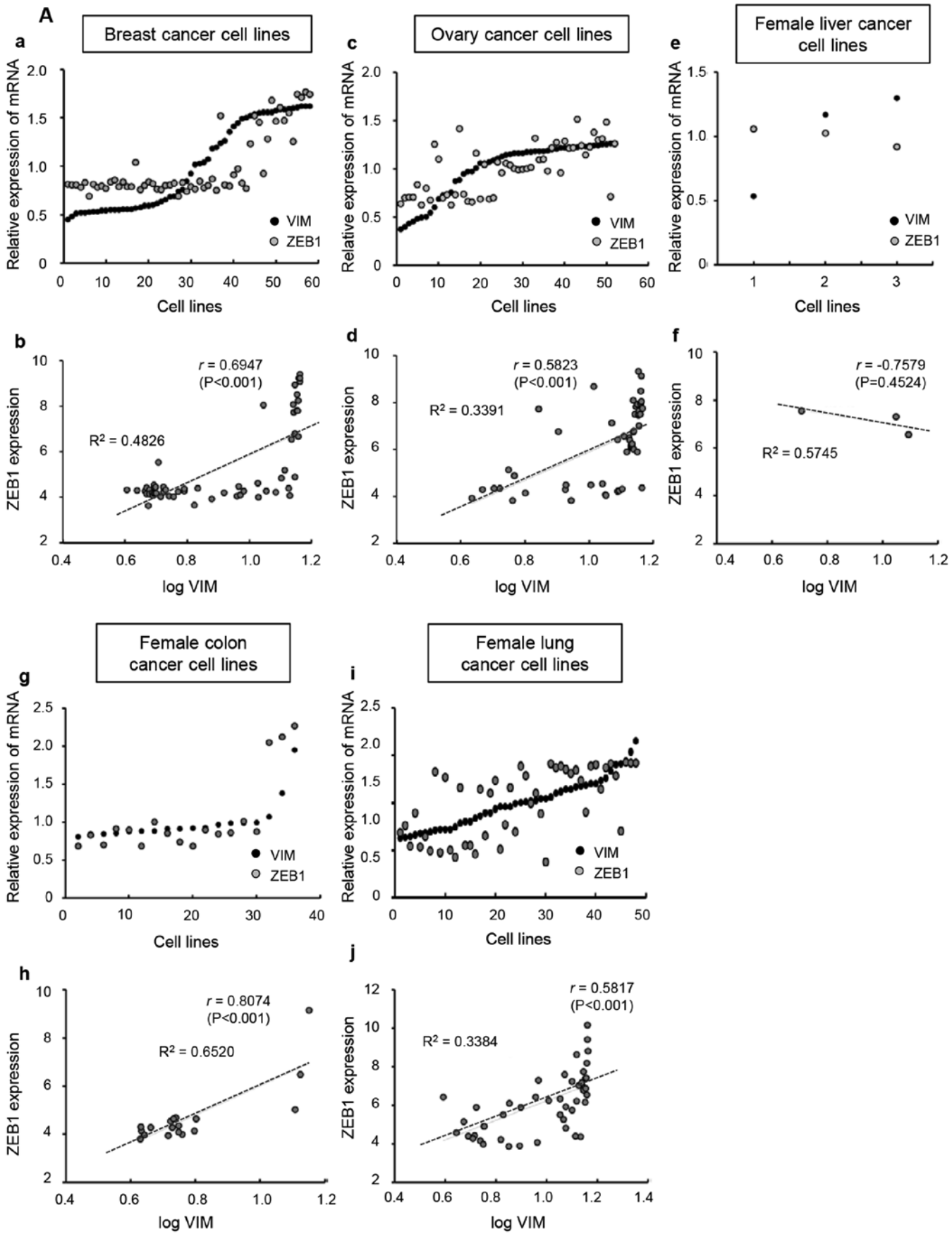

Figure 5. Broad-Novartis Cancer Cell Line Encyclopedia database analysis for mRNA levels of VIM and ZEB1 in cancer cell lines. Each graph presents a trend line with its R^2 value (the coefficient of determination) and r value (Pearson correlation coefficient). (A) Female-derived cancer cell lines. (a, c, e, g and i) the mRNA value of VIM or ZEB1 in each cell line was divided by the average value of mRNA expression and plotted. (b, d, f, h and j) ZEB1 expression levels were plotted against the logarithmic values of VIM levels. VIM, vimentin; ZEB1, zinc finger E-box binding homeobox.

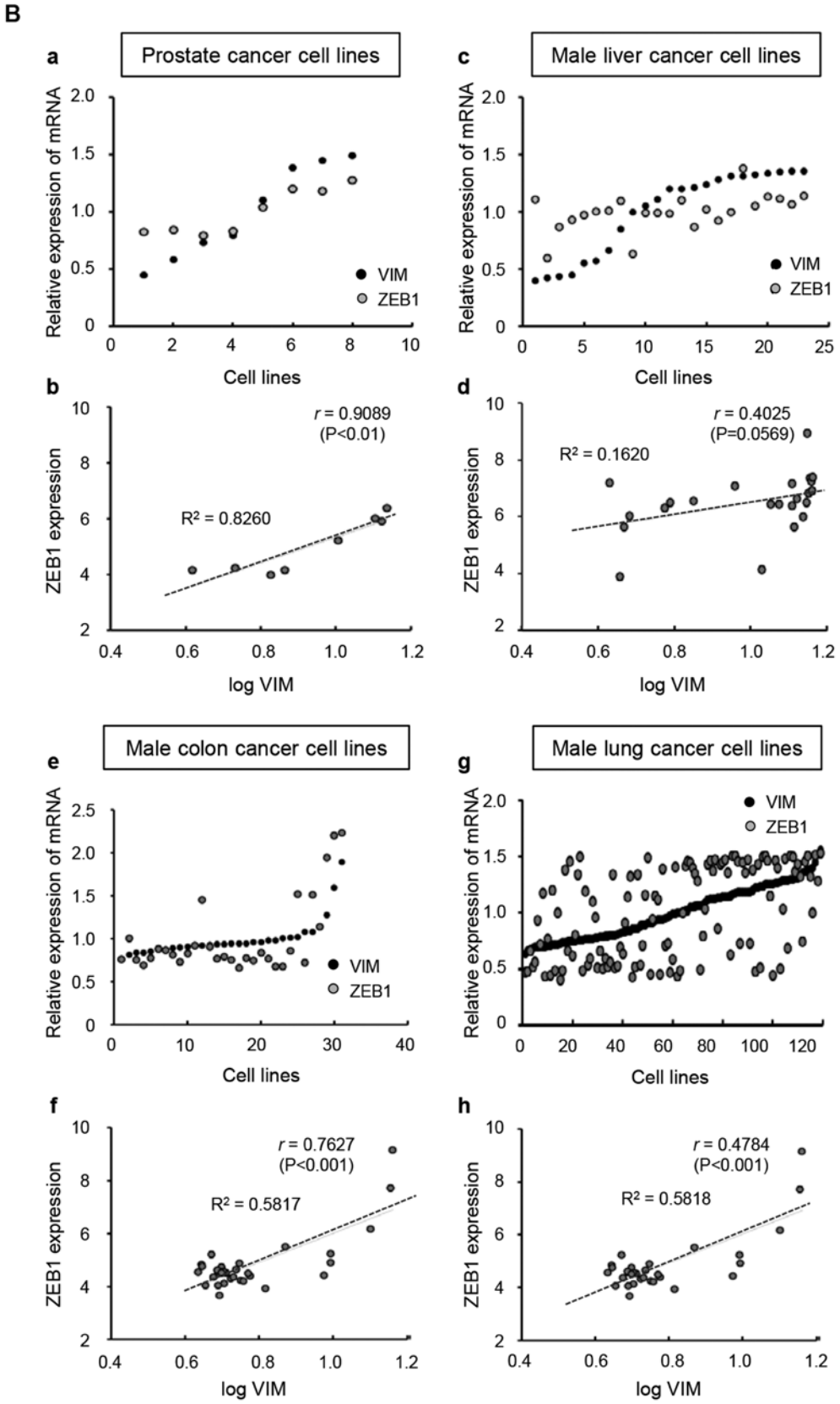

Figure 5. Continued. (B) Male-derived cancer cell lines. (a, c, e and g) the mRNA value of VIM or ZEB1 in each cell line was divided by the average value of mRNA expression and plotted. (b, d, f and h) ZEB1 expression levels were plotted against the logarithmic values of VIM levels. VIM, vimentin; ZEB1, zinc finger E-box binding homeobox.

The correlation between VIM and ZEB1 revealed a positive linear relationship in the female-derived (Fig. 5A), except female liver cancer cell lines, and male-derived (Fig. 5B) cell lines. The r values of log VIM with ZEB1 in female

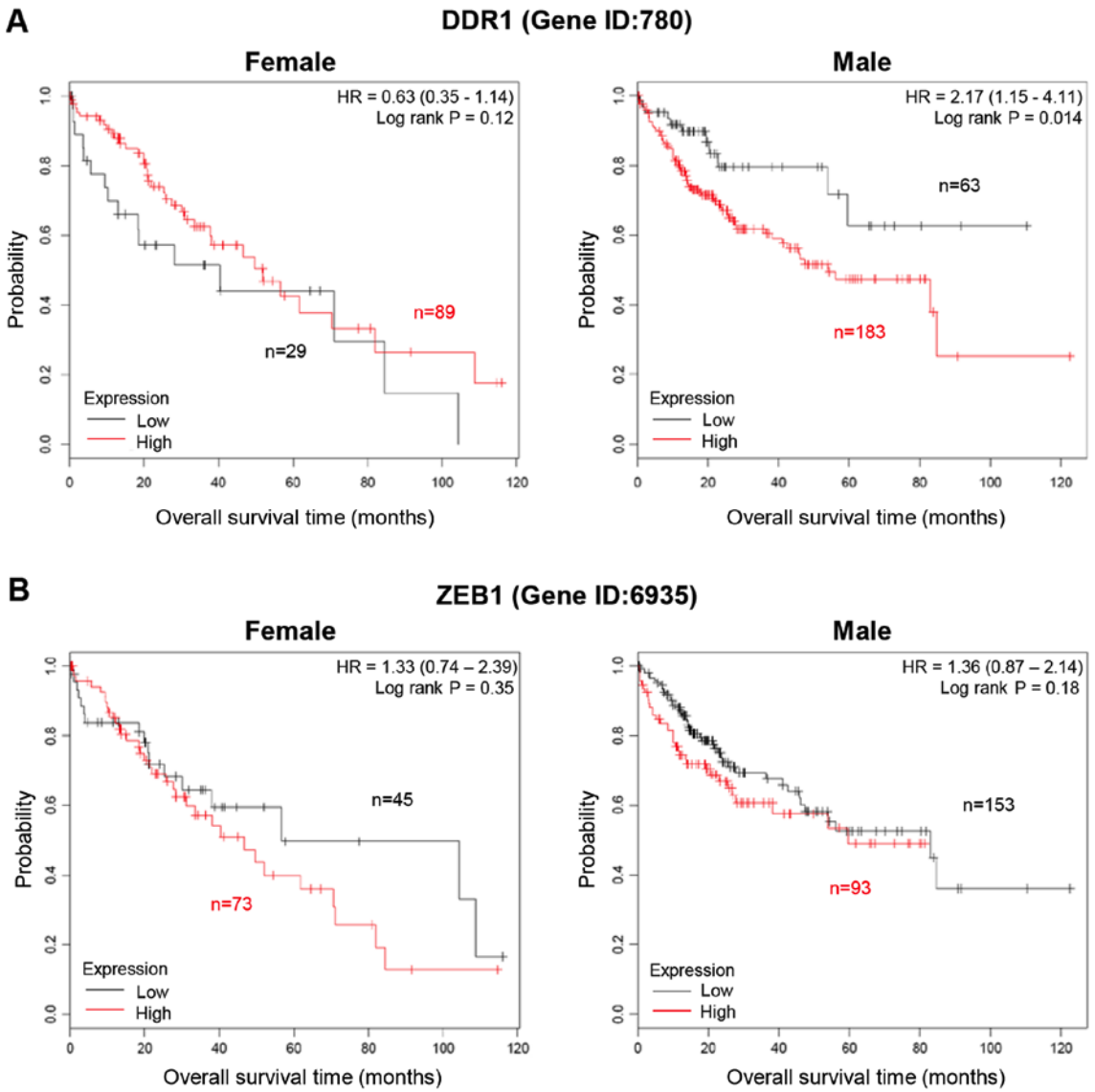

Figure 6. Kaplan-Meier analysis assessing DDR1 or ZEB1 level and overall survival time of patients with liver cancer. Kaplan-Meier curves plotted comparing overall survival with (A) DDR1 or (B) ZEB1 expression levels in female and male patients with liver cancer groups. DDR1, discoidin domain receptor; ZEB1, zinc finger E-box binding homeobox; HR, hazard ratio.

breast-, ovary-, liver-, colon- and lung-derived cell lines were 0.6947 (P<0.001), 0.5823 (P<0.001), -0.7579 (P=0.4524), 0.8074 (P<0.001) and 0.5817 (P<0.001), respectively. The r values of log VIM and ZEB1 in male prostate-, liver-, colon- and lung-derived cell lines were 0.9089 (P<0.01), 0.4025 (P=0.0569), 0.7627 (P<0.001) and 0.4784 (P<0.001), respectively. Taken together, the data from the CCLE databases revealed that there were no sex-specific differences in the correlation between CDH1 and ZEB1, as well as in that between VIM and ZEB1.

Relative correlation of mRNA expression between EMT-associated genes and ZEB1 by Pearson and Spearman correlation coefficients. In order to evaluate the relative correlation of mRNA expression between EMT-associated genes and ZEB1, both the Pearson and Spearman correlation coefficients were calculated (Table I). Out of the total 27 correlation sets, 5 sets revealed differences in the correlation coefficients of Pearson and Spearman. The expression levels of CDH1 and ZEB1 were negatively correlated, and those of VIM and ZEB1 were positively correlated, regardless of the sexual origin of cancer cell lines (Table I). In contrast, the negative correlation between DDR1 and ZEB1 in cell lines was observed in a female-specific manner in cancer cell lines such as female-specific organ cancers and liver cancer. These data revealed that the negative or positive correlation between DDR1 and ZEB1 indicated a sex-biased correlation in liver cancer cell lines specifically.

Analysis of TCGA database reveals a sex-biased difference in the correlation between DDR1 expression level and overall survival in female or male patients with liver cancer. The transcriptomic datasets, such as GEO, EGA and TCGA, were analyzed for the gene expression levels of DDR1/ZEB1 and overall survival time in female and male patients with liver cancer (40,41). The Kaplan-Meier survival curves were obtained using the Kaplan-Meier Plotter program (Fig. 6). In Fig. 6A, high expression level of DDR1 revealed an improved

survival time in female patients with liver cancer (Hazard ratio, HR=0.63, logrank P=0.12). In male patients with liver cancer however, high levels of DDR1 revealed a worse survival time (HR, 2.17; logrank P=0.014). High levels of ZEB1 revealed a worse overall survival time in both female and male patients with liver cancer although not specific, logrank P=0.35 and 0.18, respectively. (Fig. 6B). These data suggest a sex-biased difference in correlation between DDR1 expression pattern and overall survival time in patients with liver cancer although not all of the KM plots analyzed showed demonstrated significant results.

Discussion

The differences in incidence, progression and mortality rates of cancer between each sex have been well documented (1-3). Sex differences in cancer have recently been characterized at the molecular level (14). The present study aimed to elucidate the molecular basis for sex-biased gene expression in cancer. The mRNA expression of EMT-associated genes including CDH1, VIM, DDR1 and ZEB1 in female- and male-derived cancer cell lines were investigated experimentally (Fig. 1) and using CCLE database analysis (Figs. 3-5) of human cancer cell lines and Kaplan-Meier survival curves of female or male patients with liver cancer (Fig. 6). The present study revealed that the expression levels of these genes in cancer cell lines were not sex-biased, which was consistent with a previous report (14).

In order to evaluate the relative correlation of mRNA expression between EMT-associated genes and ZEB1, the Pearson and Spearman correlation coefficients were calculated (Table I). As presented in Table I, the expression levels of CDH1 and ZEB1 were negatively correlated, and those of VIM and ZEB1 were positively correlated, regardless of sexual origin of cancer cell lines (Table I). However, two correlation coefficients of ZEB1 and logVIM were calculated as a negative value only in female liver cancer cell lines. This may be due to the low number of cell lines used in the study because there are relatively few studies on female liver cancer compared to many studies on the risk of male liver cancer. Therefore, further studies on female liver cancer are needed.

In contrast, the negative correlation between DDR1 and ZEB1 in cancer cell lines was observed in a female-specific manner in cell lines of female-specific organs, such as breast and ovary cancer, and liver cancer, where the significant sex difference was reported (4-6). The correlation between DDR1 and ZEB1 were negative in both male- and female-derived cancer cell lines of colon and lung cancer. Notably, recent reports have revealed that the incidence and mortality of colon and lung cancer were more likely to be associated with socioeconomic development, and not with sex (44,45). The sex-biased correlation between DDR1 and ZEB1 in liver cancer cell lines suggests that the regulatory mechanism of gene expression, rather than the expression itself, may be affected by sex.

Invasiveness is an important step in the malignant transformation of cells (46). CDH1, VIM, DDR1 and ZEB1 are associated with the invasive phenotype of cancer cells (27,30,36,47-49). To simplify the CDH1-, VIM- or DDR1-to-ZEB1 ratios, the CDH1, VIM, or the DDR1 indices were determined in the present study. The data suggest that the DDR1 index may be a sex-specific indicator of the invasive potential of cancer cell lines, while the correlation between the CDH1 index or the VIM index and relative invasion was not affected by the origin of sex, and is therefore not a suitable indicator (Fig. 2).

The correlation between DDR1 index and invasiveness may be associated with sex. Genetic factors, environmental causes and sex hormones may contribute to sex disparity in susceptibility to cancer (1,3,12,13). As all cell lines used in research can be assigned to a sex (50), cells derived from females or males are different in cellular biochemistry and physiology due to the presence of sex chromosomes (51). The effect of hormones on the sex-specific gene expression of a cell line has been controversial. Several studies have suggested that hormones affect the gene expression profiles of a cell line (12,13), while other studies have demonstrated that the sex-specific genetic differences in cell lines existed prior to hormone exposure (52-54). It has been demonstrated in a number of genes that sexually dimorphic gene expression was associated with the presence of the X or Y chromosomes, and not with hormones (55,56). As the cell lines were not treated with hormones in the present study, it can be suggested that the sex-specific difference of correlation between DDR1 and ZEB1 expression may not be due to the effects of hormones. The human clinical data from GEO, EGA and TCGA databases were also analyzed for the expressions of DDR1 and ZEB1 in patients with liver cancer. These human data reflect the effect of hormones. Although the databases do not directly address the cause of these differences, several studies using the databases mentioned hormones as the cause of these differences in liver cancer (57,58).

A recent study revealed the sex-biased molecular signatures affecting cancer (14). The present study demonstrated sex-biased differences in the correlation of EMT-associated gene expression. Comparative studies using gene-expression data of various types of cancer reported that the expression patterns of certain genes were specific to individual cancers depending on the type (4,59,60). The results from the present study demonstrate that the negative correlation of DDR1 and ZEB1 expression was observed only in certain types of cancer, which may be explained by the cancer-specific expression patterns of certain genes. A sex-specific correlation was observed between DDR1 and ZEB1 in cell lines derived from liver cancer, which is already known to be sex-biased (4,5), whereas it was not detected in cell lines from colon or lung cancers in which the obvious sex dependence has not previously been observed (10,11). The results of the present study imply that the sex-biased regulation of gene expression may contribute to the sex differences in the susceptibility and progression of cancer. The present study suggests a potential application of the correlation between DDR1 and ZEB1 for investigating sex-biased cancers, such as liver cancer. These results may provide useful information for establishing sex-specific strategies for diagnosis and therapy of cancer.

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Availability of data and materials

The datasets used and analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

SYK and SL designed and conducted the study and wrote the manuscript. EL, HL and JYS conducted the study and helped to write the manuscript. JJ and SGK designed the study and helped to analyze the data. AM conceived and designed the study as corresponding author of the manuscript. All authors reviewed and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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