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ORIGINAL REPORT

ERCC5 Is a Novel Biomarker of Ovarian Cancer Prognosis

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ABSTRACT

Purpose
To identify a biomarker of ovarian cancer response to chemotherapy.

Patients and Methods
Study participants had epithelial ovarian cancer treated with surgery followed by platinum-based chemotherapy. DNA and RNA were isolated from frozen tumors and normal DNA was isolated from matched peripheral blood. A whole-genome loss of heterozygosity (LOH) analysis was performed using a high-density oligonucleotide array. Candidate genomic areas that predicted enhanced response to chemotherapy were identified with Cox proportional hazards methods. Gene expression analyses were performed through microarray experiments. Candidate genes were tested for independent effects on survival using Cox proportional hazards models, Kaplan-Meier survival curves, and the log-rank test.

Results
Using a whole-genome approach to study the molecular determinants of ovarian cancer response to platinum-based chemotherapy, we identified LOH of a 13q region to predict prolonged progression-free survival (PFS; hazard ratio, 0.23; $P = .006$). *ERCC5* was identified as a candidate gene in this region because of its known function in the nucleotide excision repair pathway, the unique DNA repair pathway that removes platinum-DNA adducts. We found LOH of the *ERCC5* gene locus and downregulation of *ERCC5* gene expression to predict prolonged PFS. Integration of genomic and gene expression data shows a correlation between 13q LOH and *ERCC5* gene downregulation.

Conclusion
ERCC5 is a novel biomarker of ovarian cancer prognosis and a potential therapeutic target of ovarian cancer response to platinum chemotherapy.

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INTRODUCTION

Epithelial ovarian cancer is one of the most platinum-sensitive solid malignancies, with 70% of patients achieving a complete clinical remission after front-line therapy with a platinum-based chemotherapeutic regimen.[1] However, despite this initial success, approximately 50% of patients will develop recurrent disease within 3 years of diagnosis.[2] Paradoxically, although most patients initially respond to platinum chemotherapy, the majority eventually die from chemotherapy-resistant disease.[3,4] The identification of molecular agents that effectively target the mechanisms of chemotherapy resistance could represent a significant advancement in our ability to treat these often fatal malignancies.[5]

In this study, we approached the question of response to platinum chemotherapy through an analysis of the genetic changes occurring in ovarian cancer. All patients in the analysis underwent standard surgical cytoreduction followed by an adjuvant platinum-based regimen, allowing us to probe for potential genetic markers of platinum-sensitivity. Using a whole-genome approach, we found loss of heterozygosity (LOH) at a 13q region to strongly predict prolonged progression-free survival (PFS). Within this region, we identified the *ERCC5* gene, which encodes the XPG protein. XPG is a key member of nucleotide excision repair (NER) pathway, the DNA repair mechanism responsible for removing bulky DNA adducts. We hypothesized that a loss of XPG function would be correlated with diminished ability to repair platinum-induced DNA damage, enhanced platinum-sensitivity, and prolonged PFS. We found associations between LOH of 13q, which is the *ERCC5* gene locus, and prolonged PFS, *ERCC5* downregulation and prolonged PFS, as well as a correlation between LOH of 13q and *ERCC5* gene downregulation.

Our findings lend support to prior work that has suggested the importance of the NER pathway in response to platinum-based chemotherapy and suggests *ERCC5* (XPG) as a novel candidate biomarker of ovarian cancer response to platinum chemotherapy. Further work on this pathway may validate XPG as a diagnostic marker and/or lead to the development of a therapeutic agent that specifically targets XPG activity for the sensitization of platinum-resistant malignancies.

PATIENTS AND METHODS

Patient Samples

All patient samples were collected at Cedars-Sinai Medical Center using protocols approved by the Cedars-Sinai institutional review board. After patients provided informed consent, fresh tumor tissue was snap-frozen in liquid nitrogen and stored in a −80°C freezer. Study participants were treated with surgical cytoreduction followed by platinum-based chemotherapy regimen and had corresponding clinical and follow-up information.

DNA was isolated from frozen tumor samples using the Qiagen DNeasy Tissue Protocol (QIAGEN, Valencia, CA). RNA was isolated from frozen tumor samples using the RNeasy kit (QIAGEN). Genomic DNA was isolated from matched peripheral-blood samples using a standard phenol chloroform extraction method. DNA and RNA quantities were measured with the Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). RNA quality was determined through separation by capillary electrophoresis on the Agilent 2000 Bioanalyzer (Agilent Technologies, Foster City, CA). Microarray analysis was performed with high-quality RNA, defined as an RNA integrity number greater than 8.

DNA Genomic Analysis Using GeneChip Mapping 50K High-Density Oligonucleotide Array

Array experiments were performed using standard Affymetrix protocols (Affymetrix, Santa Clara, CA). For each sample, 250 ng of total genomic DNA was digested with *XbaI* restriction enzyme and ligated to common adaptors, which allowed for polymerase chain reaction (PCR) amplification of the entire genome using a single pair of primers. PCR products were digested, labeled, and hybridized to the 50K *Xba* high-density oligonucleotide microarray, which contains a marker distance of approximately 50 kb between single-nucleotide polymorphisms.

GeneChip Data Analysis

Genotype calls were processed using the Copy Number Analyzer for GeneChip software program (CNAG; University of Tokyo, Tokyo, Japan; http://www.genome.umin.ac.jp).[6] Matched peripheral-blood DNA was used as the reference for each tumor DNA sample. Each genomic region was classified as normal, amplified, deleted, or as a region of copy-neutral LOH (also known as uniparental disomy). Summary graphs were generated with STATA v8 (STATA Corp, College Station, TX) to graphically display the range and locations of each genetic abnormality.

A discrete variable was created for each genomic block affected by LOH in at least 20% of cases. In all, 106 LOH variables were defined across the tumor genome and each was tested as a predictor affecting PFS. Cox proportional hazards methods were used to determine hazard ratios (HR) for each variable. Associations were reported if the two-sided *P* values were less than .05. Multivariate Cox models were generated to control for the effects of confounding factors. As this was an exploratory analysis, corrections were not made for multiple testing.

Identification of Candidate Genes

Genomic LOH regions were found to predict prolonged PFS. These regions were hypothesized to harbor important tumor suppressor genes that mediate response to platinum-based chemotherapy. Candidate genes were identified through a direct link from the CNAG chromosome viewer to the University of California at Santa Cruz Genome Browser Build 17 (hg17) Human May 2004 assembly, based on the National Center for Biotechnology Information Build 35 (University of California at Santa Cruz, Santa Cruz, CA; http://genome.ucsc.edu/ index.html). Predicted and reference sequence genes were investigated to narrow the candidate gene list.

LOH Analysis at Candidate Gene Locus

ERCC5, a DNA repair gene from the NER pathway, was identified as a biologically plausible modulator of ovarian cancer response to platinum-based chemotherapy. The LOH status at the *ERCC5* gene locus was evaluated in 44 clinical ovarian cancer samples to determine its specific effect on ovarian cancer prognosis. All were papillary serous histology, stages IIC to IV; 10 were common to the GeneChip data set. For each sample, a dinucleotide repeat polymorphism located within an intron of the *ERCC5* gene was amplified in tumor and matched normal DNA. Previously published primer sequences[7] were lengthened to enhance the specificity of the reaction (Forward-Fam labeled 5′GCA ATG ACT CGG TAT TGG CTA AT 3′; Reverse 5′ GAT GCT AAC AAG TGG GTG GAA T 3′). PCR was performed with 15-μL reactions containing 20 ng of genomic DNA, 20 pmole of each primer, 0.75 μL of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), and 2× dNTP (Epicenter, Madison, WI) on a GeneAmp PCR System 9700 (Applied Biosystems). PCR products were verified on 2% agarose gel and run in the University of California at Los Angeles genotyping core facility. LOH was determined in samples with a homozygous genotype in tumor DNA and a heterozygous genotype in the matched normal DNA.

Gene Expression Analysis

The significance of expression of *ERCC5* and other *ERCC* gene expression were tested using high-quality RNA extracted from 90 ovarian cancer samples. Microarray analysis was performed on the Agilent Human 1A V2 chip. Samples were labeled using the Agilent Low RNA Input Fluorescent Linear Amplification Kit. Each individual sample was labeled with cyanine-5 and characterized by comparison to a mixed reference pool labeled with cyanine-3. The mixed reference pool consisted of equal amounts of cRNA from 106 clinical samples from the tumor bank, ranging from benign to malignant, and chosen to be representative of the range of histologic pathologies occurring in the female reproductive tract. An Agilent Scanner and the Agilent Feature Extraction software version 7.5 were used to read the microarray slides and calculate gene expression values. Gene expression values of the candidate genes (*ERCC1-ERCC6, ERCC8*) were exported to STATA and linked to clinical data. The threshold for downregulation was defined as log(ratio) less than 0 with a *P* value of less than .05. The *P* values were determined according to the Agilent error model with the feature-extracted data imported into Resolver. Cox proportional hazards models, Kaplan-Meier survival curves, and the log-rank test were used to determine the effects of *ERCC* gene expression on PFS.

Quantitative PCR Validation of Gene Expression

Microarray gene expression data were validated by quantitative real-time PCR performed using the SYBR Green method (Invitrogen, Carsbad, CA) with standard curves on iCycler (Bio-Rad, Hercules, CA). RNA was converted to cDNA using the QuaniTect Reverse Transcription kit (QIAGEN). PCR reactions were performed in 96-well plates with 12.5 μL 0f SYBR Green, 0.5 μL of primer, and cDNA in a total reaction volume of 25 μL. *ERCC5* was amplified with the following primers: Forward CAGACACAGCTCCGAATTGA; Reverse TTCTGGGTTTTTCGTTTTGC. Expression of ERCC5 was normalized by 18srRNA subunit expression with the following primers: Forward CGCCGTGCCTACCATGGTGAC; Reverse CTTGGATGTGGTAGCCGTTTCTCA.

RESULTS

Study Overview and Patient Characteristics

A whole-genome analysis of the genetic changes occurring in the DNA of 20 tumors was performed to identify candidate regions that predicted improved response to treatment. Effect of LOH at the candidate gene locus was analyzed in 52 total samples. Association of RNA expression levels with prognosis was tested in 90

tumors. Patient characteristics among the three data sets are listed in Table 1. All patients were treated with initial cytoreductive surgery followed by adjuvant chemotherapy with a platinum-containing regimen. PFS was defined as the time from date of primary cytoreductive surgery to the date of first clinical evidence of recurrence. Overall survival was defined as the time from date of primary cytoreductive surgery to the date of death or censored at the date of last follow-up.

Characterization of Genomic Abnormalities in Ovarian Cancer DNA

Appendix Figure A1A (online only) is a representative example of how the visual output from the CNAG software program was converted into a color-coded graphical representation of genetic changes (amplification, deletion, or uniparental disomy). This method allowed us to summarize the genetic heterogeneity and complexity occurring over the 20 ovarian cancer samples (Appendix Fig A1B).

Table 1. Patient Characteristics at Diagnosis

Characteristic	Whole Genome Analysis (n = 20)		LOH Analysis (n = 54)		RNA Expression Analysis (n = 90)	
	No. of Patients	%	No. of Patients	%	No. of Patients	%
Age, years						
Median	62		58		59	
Range	45-72		43-82		41-82	
PFS, months						
Median	14		20		14	
Range	0.1-39.2		2.9-112.6		2.9-192.3	
OS, months						
Median	42.9		45		38.3	
Range	11.5-90.5		5.3-116.4		5.3-192.3	
Stage						
I	0	0	0	0	5	5
II	2	10	3	6	6	7
III	14	70	37	68	63	70
IV	4	20	14	26	16	18
Grade						
2	2	10	3	6	9	10
3	18	90	51	94	81	90
Histology						
Papillary serous	20	100	54	100	81	90
Other epithelial	0	0	0	0	9	10
Tumor site						
Ovarian	15	75	43	79	77	86
Fallopian tube	0	0	2	4	2	2
Primary peritoneal	5	5	9	17	11	12
BRCA mutation status						
BRCA1 carrier	3	15	8	15	10	11
BRCA2 carrier	2	10	6	11	8	9
BRCA1 and *BRCA2*	1	5	1	2	1	1
Negative/unknown	14	70	39	72	71	79
Cytoreductive surgery						
Optimal (< 1 cm)	19	95	45	83	72	80
Suboptimal (≥ 1 cm)	1	5	8	15	10	11
Unknown	0	0	1	2	8	9
Adjuvant chemotherapy						
Carboplatin/paclitaxel	18	90	48	89	67	74
Cisplatin/cyclophosphamide	2	10	2	4	18	20
Other platinum regimen	0	0	4	7	5	6
Clinical response						
Complete response	17	85	42	78	72	80
Partial response/stable	3	15	11	20	17	19
Progression	0	0	1	2	1	1
Pathologic response						
Second look negative (pCR)	8	40	14	26	25	28
Second look positive	9	45	15	28	24	27
Not evaluated	3	15	24	46		

Abbreviations: LOH, loss of heterozygosity; PFS, progression-free survival; OS, overall survival; pCR, pathologic complete response.

A similar summary graph demonstrates the frequency of LOH in the ovarian tumor genomes (Appendix Fig A1C). The LOH data were further summarized in a frequency plot demonstrating the proportion of cases affected by LOH at each genomic locus (Appendix Fig A1D).

Predictors of Prolonged PFS

LOH blocks on chromosomes 2, 5, 13, and 22 were found to predict prolonged PFS (point estimates for HRs ranging from 0.23 to 0.36; Table 2), suggesting a survival benefit owing to loss of function of genes in the regions. Adjusted HRs were calculated for each LOH block to account for the potential confounding effects of various clinical variables. LOH of the 13q (54 to 102 MB) block was found to retain a highly significant independent association with prolonged PFS after adjustment for various confounders (Table 2).

A Candidate Gene That May Predict Response to Platinum Chemotherapy

The 48-MB region on 13q (54 to 102 MB) contains 73 genes (Appendix Table A1, online only), including *ERCC5*. We felt this gene deserved further study given its role in the NER pathway, the unique DNA repair pathway that allows cells to remove platinum adducts from DNA. We hypothesized that LOH of this 13q region leads to downregulation of *ERCC5* levels, a diminished capacity of tumor cells to recover from platinum-based chemotherapy (enhanced chemotherapy sensitivity), and prolonged PFS.

Effect of LOH of ERCC Gene Locus on Survival

The LOH status of the *ERCC5* gene locus was determined through genotyping of a dinucleotide repeat polymorphism (DRP) in 44 samples. Fourteen genotypes (32%) were noninformative because of a homozygous genotype in both normal and tumor DNA. Genotype data were available from 20 samples by GeneChip analysis. Ten samples were genotyped by both methods, resulting in 54 total samples genotyped. Among the 10 overlapping samples, six genotypes were correlated, two were noninformative on DRP analysis, and two were contradictory on the basis of the finding of LOH on GeneChip but no LOH on DRP analysis. In the four noncorrelating samples, LOH status was analyzed based on GeneChip data as a result of the self-validating nature of the genotyping of multiple single-nucleotide polymorphisms with the whole-genome approach.

Among the 54 samples genotyped, 29 (54%) had no LOH, 13 (24%) had LOH, and 12 (22%) were noninformative. LOH at the *ERCC5* locus (13 of 42) demonstrated a trend toward improved PFS (Fig 1A). A subset analysis limited to the stage IIC to IIIC papillary serous tumors (nine of 29) demonstrated a significant improvement in PFS (Fig 1B).

At the time of second-look surgery, patients with LOH at the *ERCC5* locus were significantly more likely to have a pathologic complete response (five of six; 83%) than those without LOH (two of eight; 25%; $P = .03$).

Effect of ERCC Gene Expression on Survival

Gene expression levels of the various *ERCC* genes (*ERCC1* through *ERCC6*, *ERCC8*) were analyzed for influences on survival in a data set of 90 patients. PFS (Fig 1C) and OS (Fig 1D) were both prolonged among patients with *ERCC5* downregulation in the tumors. This effect was not seen for *ERCC1*, *ERCC2*, *ERCC3*, *ERCC4*, *ERCC6*, or *ERCC8* downregulation (Appendix Figs A2 through A7, online only). Microarray gene expression analysis was validated using quantitative real-time PCR (Fig 2).

Table 3 demonstrates the results from Cox proportional hazards regression models. On univariate analysis, a beneficial impact on PFS is seen with *ERCC5* downregulation (HR = 0.44; $P = .01$), but not with downregulation of any other single *ERCC* gene. On multivariate analysis, *ERCC5* downregulation retains an independent beneficial impact on PFS (HR = 0.49; $P = .03$).

Correlation Between ERCC5 LOH and Gene Expression Levels

Forty tumors with DNA and RNA data were analyzed for correlation between LOH of the 13q (*ERCC5*) locus and *ERCC5* expression

Table 2. Genomic LOH Loci Correlating With Significantly Improved PFS

Variable	n*	Crude HR	Crude 95% CI	Crude P	Adjusted† HR	Adjusted† 95% CI	Adjusted† P	Adjusted‡ HR	Adjusted‡ 95% CI	Adjusted‡ P
Genomic LOH loci										
2p: 100,819-5,524,537	5	0.28	0.08 to 0.99	.04	0.40	0.10 to 1.55	.19	0.29	0.08 to 1.02	.06
5q: 57,385,834-63,071,109	8	0.30	0.12 to 0.88	.03	0.27	0.07 to 1.00	.05	0.31	0.10 to 0.93	.03
5q: 73,164,783-81,608,220	10	0.32	0.12 to 0.88	.03	0.35	0.11 to 1.11	.08	0.34	0.12 to 0.95	.04
5q: 81,608,220-95,277,667	9	0.36	0.13 to 1.00	.05	0.36	0.11 to 1.15	.09	0.38	0.13 to 1.07	.06
13q: 18,042,610-54,217,914	8	0.24	0.08 to 0.71	.01	0.29	0.08 to 1.06	.06	0.25	0.08 to 0.75	.01
13q: 54,217,914-102,557,751	9	0.23	0.08 to 0.65	.006	0.24	0.07 to 0.88	.03	0.24	0.08 to 0.69	.008
13q: 102,557,751-114,051,456	8	0.24	0.08 to 0.71	.01	0.29	0.08 to 1.06	.06	0.25	0.08 to 0.75	.01
22q: 26,083,853-33,362,998	8	0.29	0.10 to 0.86	.03	0.35	0.10 to 1.24	.1	0.30	0.10 to 0.92	.04
22q: 44,485,638-48,983,486	10	0.31	0.12 to 0.86	.03	0.46	0.13 to 1.57	.2	0.34	0.12 to 0.93	.04
Clinical factors										
Optimal cytoreduction		0.05	0.003 to 0.86	.04						
Stage		1.64	0.80 to 3.35	.18						
Germline *BRCA* mutation		0.55	0.28 to 1.08	.09						

Abbreviations: LOH, loss of heterozygosity; PFS, progression-free survival; HR, hazard ratio.
*n represents number of cases of 20 with LOH of the genomic region.
†Adjusted for stage, optimal cytoreduction, and germline *BCRA* mutation status.
‡Adjusted for optimal cytoreduction.

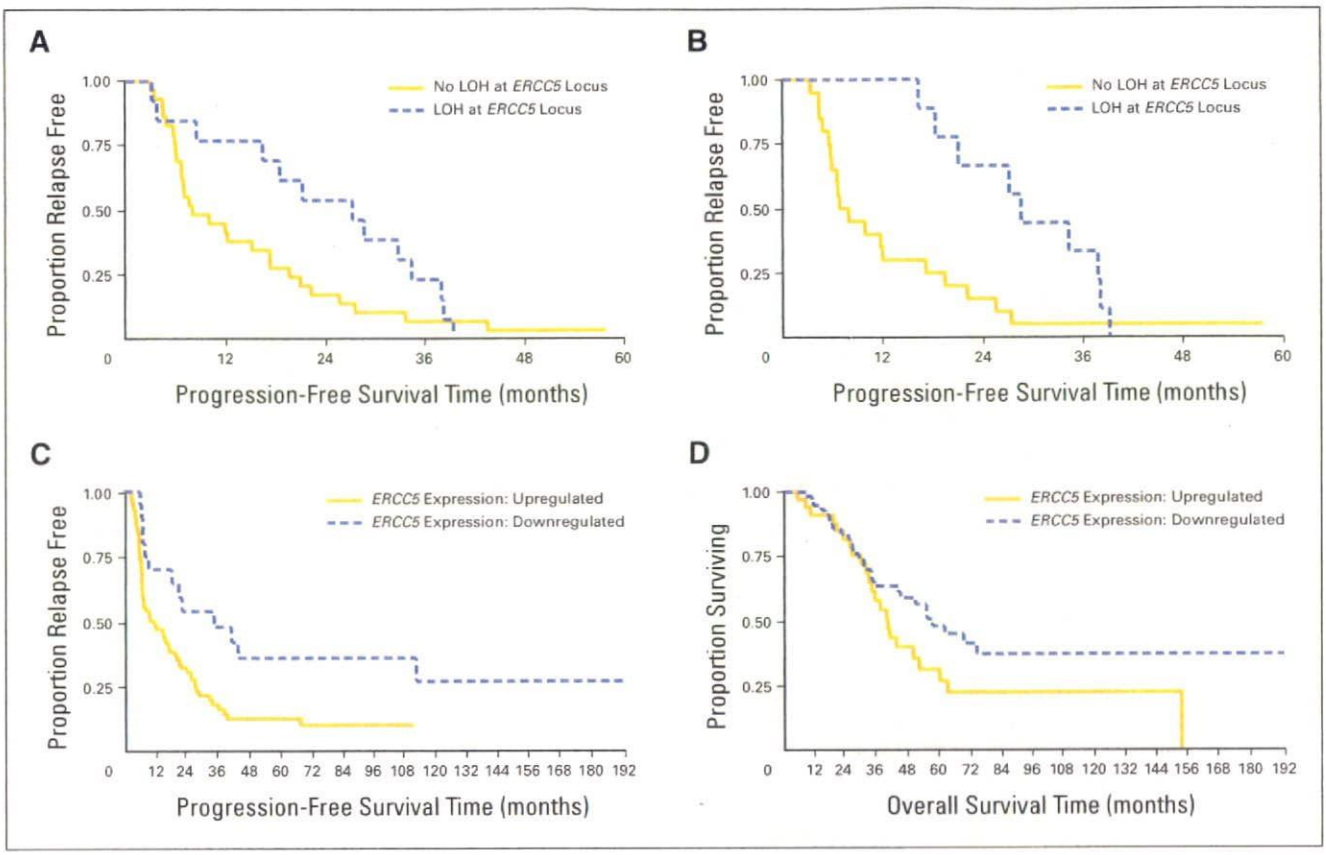

Fig 1. Survival differences based on *ERCC5* loss of heterozygosity (LOH) and gene expression. (A) Trend toward improved progression-free survival (PFS) among patients with LOH of *ERCC5* gene locus ($P = .1$) and (B) improved PFS in the subset of patients with stage IIC to IIIC disease ($P = .01$). (C) Improved PFS ($P = .004$) and (D) overall survival ($P = .08$) among patients with downregulation of *ERCC5* gene expression.

levels. Expression levels are lower in the group of tumors with LOH (mean fold change, −0.59; median −1.12) compared with the tumors without LOH (mean fold change, +0.84; median, +1.03; $P = .08$). This suggests possible biologic validity of *ERCC5* as a target gene within the 13q region.

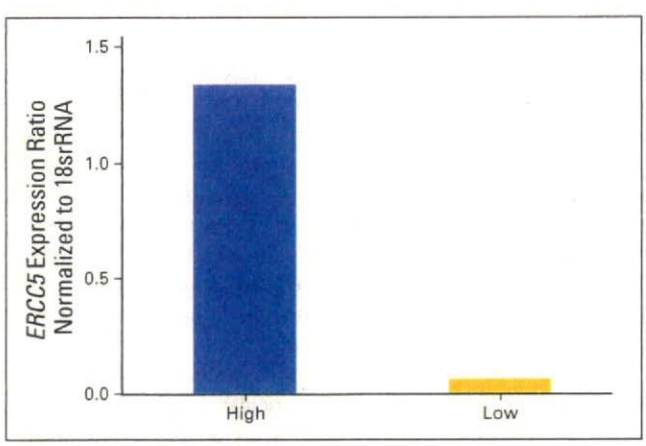

Fig 2. Quantitative polymerase chain reaction (PCR) validation of *ERCC5* gene expression. Mean differential quantitative PCR gene expression between cases with high (n = 11) and low (n = 8) *ERCC5* expression on microarray analysis ($P = .06$).

DISCUSSION

Our study identifies *ERCC5* as a novel candidate biomarker of ovarian cancer sensitivity to platinum chemotherapy. This conclusion is supported on several different levels. We found LOH of the 13q locus containing *ERCC5* to predict prolonged PFS among platinum-treated patients with ovarian cancer. Additional genotyping also confirmed an association between LOH of the *ERCC5* gene locus and improved survival. Furthermore, downregulation of *ERCC5* mRNA expression levels also predicted prolonged PFS in an independent data set. Finally, the correlation between LOH of the *ERCC5* genomic locus with downregulation of *ERCC5* mRNA levels among the subset of tumors with integrated genomic and gene expression data suggests possible biologic plausibility of *ERCC5* being a target gene in the 13q LOH region.

Further biologic plausibility is apparent when placing these findings in the context of previous knowledge and work. Defective DNA repair pathways allow tolerance to DNA damage, permitting an accelerated rate of mutagenesis and neoplastic transformation. This characteristic turns to the disadvantage of the cancer cell when DNA damaging cancer therapies are administered, leading to an enhanced response to treatment.[8] We have found evidence to support the hypothesis that a downregulation of *ERCC5* activity leads to enhanced platinum chemotherapy sensitivity in ovarian

Table 3. Cox Proportional Hazards Regression: Impact of Clinical and *ERCC* Gene Expression Data on Progression-Free Survival

Variable	No. of Patients	No. of Relapses	HR	95% CI	P
Univariate analysis					
BRCA mutation carrier	18	15	0.63	0.36 to 1.12	.12
Optimal cytoreduction	72	56	0.53	0.30 to 0.92	.02
Late stage, III/IV	79	70	5.92	1.86 to 18.86	.003
Serous histology	81	70	2.88	0.91 to 9.16	.07
ERCC1 downregulation	12	12	0.93	0.50 to 1.74	.84
ERCC2 downregulation	23	18	0.73	0.43 to 1.25	.2
ERCC3 downregulation	16	13	0.83	0.45 to 1.51	.5
ERCC4 downregulation	0				
ERCC5 downregulation	20	13	0.44	0.24 to 0.82	.01
ERCC6 downregulation	5	4	0.71	0.26 to 1.97	.5
ERCC8 downregulation	3	3	2.17	0.67 to 6.98	.2
Multivariate analysis					
ERCC5 downregulation			0.49	0.25 to 0.92	.03
Late stage, III/IV			5.61	1.76 to 17.9	.004
Optimal cytoreduction			0.66	0.38 to 1.16	.1

Abbreviation: HR, hazard ratio.

cancer. This is provocative when considering the function of *ERCC5* in the NER pathway, the unique DNA repair pathway that repairs DNA damage caused by platinum agents.

NER recognizes and repairs bulky, helix-distorting adducts, such as those formed by cisplatin and its analogs.[9,10] A complex of proteins assembles, binds bulky DNA damage, incises the oligonucleotide fragment containing the damaged base, and fills in the resulting gap.[11,12] Platinum-resistant cells are able to more effectively remove cisplatin-DNA adducts through the action of a functional NER pathway and thus escape apoptosis.

ERCC5 (XPG) is a structure-specific endonuclease, which participates in two incision steps that are critical to the DNA repair process. XPG cleaves the damaged DNA 3′ to the damaged site, nonenzymatically participates in the 5′ incision mediated by the XPF/ERCC1 heterodimer, and stabilizes the DNA repair complex to the damaged DNA.[13-16] XPG is critical to both subpathways of NER: transcription-coupled repair (TCR), which specifically targets and repairs DNA damage on the transcribed strand of actively expressed genes, and global genomic repair (GGR), which removes DNA damage from the remaining genome.[17] TCR and GGR each have a unique mechanism for recognizing DNA damage, then progress along a common pathway that requires XPG.[11,12]

A number of studies provide evidence for the role of the NER pathway in cellular response to platinum chemotherapy, consistently demonstrating platinum-resistance with enhanced NER activity and platinum-sensitivity with diminished NER activity. Cisplatin-resistant cells have been shown to have increased levels of *XPA* mRNA,[18] overexpression of *ERCC1* or *ERCC1/XPF*,[19] increased activity of *ERCC1/XPD*,[20] and increased *XPC* and *ERCC1* levels.[21] Hypersensitivity of some cell lines may be related to reduced expression of *XPG* or *XPA*.[9] Inhibition of *ERCC1* activity with antisense oligonucleotides enhances cisplatin sensitivity in ovarian cancer cell lines.[22] Cells with deficiencies in GGR-specific factors (XPC) display normal resistance to cisplatin, whereas cells with deficiencies in TCR-specific (CSA, CSB) or common pathway proteins (XPA, XPD, XPF, XPG) are markedly hypersensitive to cisplatin.[23] The excellent response rates of testicular cancer to cisplatin may be due to a high rate of NER deficiencies.[24]

Our study adds to this body of literature, suggesting that loss of *ERCC5* function occurs naturally during the carcinogenic process of a subset of ovarian cancers and consequently leads to inherent platinum chemotherapy sensitivity. This speculation is supported by the finding of LOH of regions harboring NER genes occurring at a higher frequency in ovarian cancers (62%) than in other solid tumors, such as colon or lung cancer.[25] Ours is not the first study to report frequent LOH of the 13q locus in epithelial ovarian cancer.[26]

Our findings have important prognostic and therapeutic implications. Tumors with dysfunctional *ERCC5* expression would be predicted to demonstrate sensitivity to platinum-based therapy. *ERCC5* (XPG) may be an appropriate target for therapeutic inhibition in platinum-resistant ovarian cancers. XPG is a critical component of the rate-limiting damage recognition/excision step of NER[27] and is expressed at lower cellular protein levels than other NER factors.[28] XPG levels are correlated with cytotoxicity to cisplatin and irofulven and with cellular NER activity,[28] potentially making it an attractive therapeutic target. A recent integrated analysis of array CGH and gene expression profiling data in testicular cancers also found *ERCC5* to be both lost and downregulated.[29] Ovarian cancer and testicular cancer share the quality of platinum chemotherapy sensitivity, and data are emerging to suggest that NER dysfunction (particularly *ERCC5*) may be another shared characteristic. Further work may lead to the development of a specific XPG inhibitor that can sensitize platinum-resistant tumors to the effects of platinum chemotherapy.

Platinum drugs demonstrate activity in a wide range of tumors, including ovarian, cervical, testicular, head and neck, and non–small-cell lung cancer,[30] but their use is often limited by the development of resistance. A number of complex pathways are involved, including decreased drug uptake into the cell, increased drug inactivation, and increased DNA repair.[31] Further insights

into these mechanisms could be used to develop rational biologic therapies that target platinum resistance.[32]

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

Cloning of genes involved in chromosomal translocations by high-resolution single nucleotide polymorphism genomic microarray

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High-resolution single nucleotide polymorphism genomic microarray (SNP-chip) is a useful tool to define gene dosage levels over the whole genome, allowing precise detection of deletions and duplications/amplifications of chromosomes in cancer cells. We found that this new technology can also identify breakpoints of chromosomes involved in unbalanced translocations, leading to identification of fusion genes. Using this technique, we found that the *PAX5* gene was rearranged to a variety of partner genes including *ETV6*, *FOXP1*, *AUTS2*, and *C20orf112* in pediatric acute lymphoblastic leukemia (ALL). The 3′ end of the *PAX5* gene was replaced by the partner gene. The PAX5 fusion products bound to PAX5 recognition sequences as strongly as wild-type PAX5 and suppressed its transcriptional activity in a dominant-negative fashion. In human B cell leukemia cells, binding of wild-type PAX5 to a regulatory region of *BLK*, one of the direct downstream target genes of PAX5, was diminished by expression of the PAX5-fusion protein, leading to repression of *BLK*. Expression of PAX5-fusion genes in murine bone marrow cells blocked development of mature B cells. PAX5-fusion proteins may contribute to leukemogenesis by blocking differentiation of hematopoietic cells into mature B cells. SNP-chip is a powerful tool to identify fusion genes in human cancers.

chromatin immunoprecipitation | dominant negative | fusion gene | PAX5 | SNP-chip

Pediatric acute lymphoblastic leukemia (ALL) is the most common malignant disease in children (1–3). It is a genetic abnormality resulting from accumulation of mutations in tumor suppressor genes and oncogenes (1–3). Fusion genes including *ETV6/RUNX1* and *E2A/PBX1* are frequently detected in pediatric ALL (1). Deletion of the *INK4A/ARF* gene (9p21) is also a common abnormality in ALL (1). However, other genetic changes remain to be elucidated in this disease.

Identification of mutated genes in ALL has evolved with improvements in technology. A very recent approach is single nucleotide polymorphism (SNP) analysis using an array based technology (4–6) that allows identification of amplifications, deletions, and allelic imbalances, such as uniparental disomy (represents doubling of the abnormal allele due to somatic recombination or duplication, and loss of the other normal allele) (7, 8). However, SNP-chip analysis is only able to detect changes of gene dosage and is unable to identify balanced translocations, which commonly occur in ALL.

Previously, we analyzed 399 pediatric ALL cases by SNP-chip analysis and found a number of genomic abnormalities, in addition to well known common alterations (9). This technique is sensitive enough to identify genes involved in start sites of deletions/duplications. Indeed, this method allowed us to identify that the *PBX1* gene was involved in start sites of duplication of 1q23 generated by der(19)t(1;19)(q23;p13) (9). Furthermore, correlation analysis of the individual genomic abnormalities suggested the presence of der(12)t(12;21)(p13;q22) and der(21)t(12;21)(p13;q22), as well as dic(9;20)(p13;q11) (9).

In this study, we found that this new technology permitted us to identify genes involved in well known unbalanced translocations including *ETV6/RUNX1*. Further, we found previously undetected fusion genes between *PAX5* and a number of other partner genes by using this technique.

Results

Genes Involved in Unbalanced Translocations Were Identified by SNP-Chip Analysis. Because SNP-chip analysis can only detect changes of gene dosage including deletions, duplications, and amplifications (Fig. 1*A*), this technique is unable to identify balanced translocations (Figs. 1*Aii*). However, when one of a pair of reciprocally translocated chromosomes is lost, SNP-chip analysis can detect this abnormality as partial deletions of involved chromosomes (Fig. 1*Aiii*). Similarly, when one of a pair of reciprocally translocated chromosomes becomes duplicated, SNP-chip can also detect this abnormality as partial duplication of the involved chromosomes (Fig. 1*Aiv*). Furthermore, high resolution SNP-chip analysis allows us to identify the genes involved in these unbalanced translocations.

To prove that SNP-chip analysis can detect unbalanced translocations and the genes involved in these translocations, we

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. EU784145, PAX5-FOXP1; EU784146, PAX5-AUTS2; EU784147, PAX5-C20orf112 short isoform; and EU784148, PAX5-C20orf112 long isoform).

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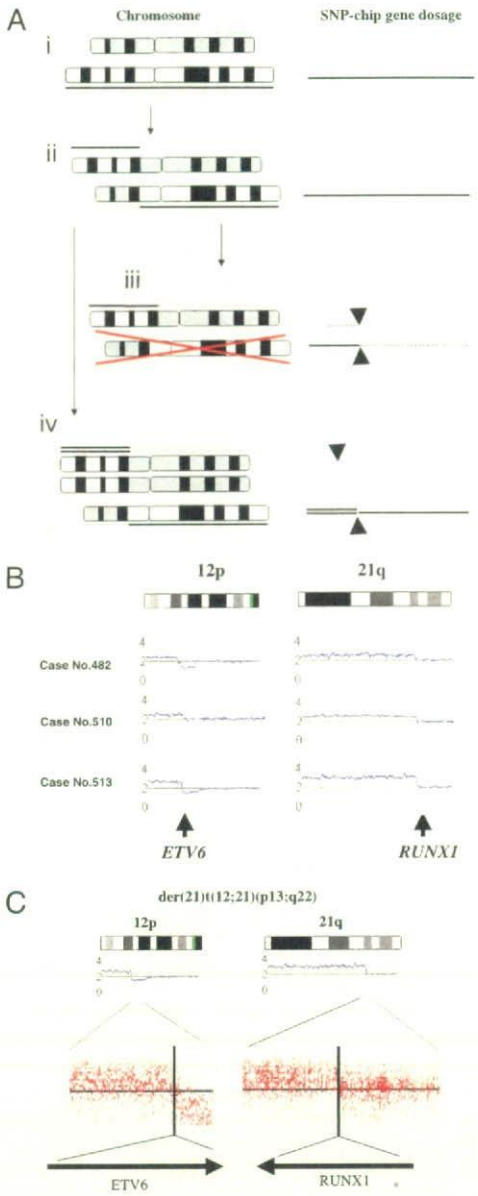

Fig. 1. SNP-chip analysis detected genes involved in unbalanced translocations. (*A*) SNP-chip analysis can identify breakpoints of translocations when one of the paired translocated chromosomes is either lost or duplicated/amplified. (*Left*) Chromosomal status. Gene dosages are indicated either above or beneath the chromosomes. (*Right*) Results of SNP-chip analysis. (*Ai*) Normal chromosomes; gene dosage is normal. (*Aii*) Reciprocal translocation; gene dosage is normal. (*Aiii*) One of the paired translocated chromosomes is lost; gene dosage is lower than normal on the left side of the upper chromosome and the right side of the lower chromosome. Arrow heads indicate the breakpoint of the translocation in each chromosome. (*Aiv*) One of the paired translocated chromosomes is duplicated; gene dosage is higher than normal on the right side of the upper chromosome and the left side of the lower chromosome. Arrow heads indicate the breakpoint of this translocation in each chromosome. (*B*) Representative cases with unbalanced translocation of der(21)t(12;21)(p13;q22). (*Left*) Start sites of duplication at 12p13 involving the *ETV6* gene. (*Right*) Start sites of duplication at 21q22 involving the *RUNX1* gene. SNP-chip data of representative cases with dup(12)(p13) and dup(21)(q22) are shown. These abnormalities were validated by FISH and/or RT-PCR (data not shown). Results of SNP-chip data were visualized by CNAG software. Lines above each chromosome show total gene dosage; level 2 indicates diploid (2N) amount of DNA, which is normal. (*C*) Magnified view of SNP-chip data. (*Upper*) Start sites of duplications at 12p13 and 21q22 are magnified. Signals of individual probe signals are shown. Vertical lines indicated the positions of start sites of duplications. (*Lower*) Genes involved in the start sites of duplications.

analyzed cases having extra copies of *ETV6/RUNX1* fusion genes generated by der(21)t(12;21)(p13;q22) (Fig. 1*B*), which were initially identified by FISH and/or RT-PCR (data not shown). SNP-chip was clearly able to identify this abnormality as duplications involving chromosome 12 and 21 (Fig. 1*B*). Further, the result of high-resolution (250k) SNP-chip clearly identified *ETV6* (12p13) and *RUNX1* (21q22) as the target genes involved in this unbalanced translocation (Fig. 1*C*).

PAX5 Gene Is Frequently Fused to Partner Genes. Our previous data showed the presence of dic(9;20)(p13;q11) in 11 cases of ALL (9), 5 of which had deletion 9p13.2-pter. These 5 cases had start sites of this deletion at 9p13.2 mapping to the *PAX5* gene (Fig. 2*A* and data not shown). This prompted us to reexamine all cases of B-ALL that had deletion of 9p [supporting information (SI) Table S1]. We found a total of 9 cases with similar start sites (9p13.2), mapping to the *PAX5* gene (Fig. 2*A* and data not shown). In 2 of these cases, simple abnormalities were detected by SNP-chip: case 514 had only del9p13.2-pter and del7q11.2-pter; case 458 had only del9p13.2-pter and dup3p13-pter (Table S1 and Fig. 2*A*). Three cases (536, 543, 572) had complex abnormalities including del9p13.2-pter and del20q11.21-qter, all with the *C20ORF112* gene within the start site of del20q (Table S1 and Fig. 2*A*). The other 2 cases (659, 767) had complex abnormalities that included *ETV6* on 12p13 (Table S1 and Fig. 2*A*).

Thus, we found four candidate partner genes fused to *PAX5* in seven cases by SNP-chip analysis; *ETV6* on 12p13 (two cases) (12), *C20orf112* on 20q11.1 (three cases), *AUTS2* on 7q11.1 (one case) and *FOXP1* on 3p13 (one case) (Fig. 2*A*). Because these translocations could lead to fusion transcripts between *PAX5* and different partner genes, the presence of the predicted fusion transcript was examined by RT-PCR using the mapping information from the SNP-chip data. RT-PCR and nucleotide sequencing data of the PCR products confirmed that the *PAX5* gene was fused to either the *ETV6* (two cases), *C20orf112* (three cases), *AUTS2* (one case), or *FOXP1* (one case) gene and transcribed into aberrant fusion messages (Fig. 2 *B* and *C*). Each fusion gene was mutually and exclusively detected in the samples studied. In one case with dic(9;20), exon 5 of *PAX5* was fused to exon 8 of *C20orf112*, and in two cases with dic(9;20), exon 8 of *PAX5* was fused to exon 3 of *C20orf112*. *PAX5/ETV6* involved exon 4 of *PAX5* and exon 3 of *ETV6*.

Cellular Localization and DNA Binding Affinity of PAX5 Fusion Products. In the *PAX5/FOXP1* fusion transcript, the amino acid coding frame of the *FOXP1* gene was not identical to that of *PAX5*, leading to a frame-shift and an early termination codon after the fusion point of these two genes (Fig. 2*D*). However, all other fusion genes were in frame and were predicted to encode chimeric proteins. Two proteins (a short and long form) with different breakpoints were predicted from the *PAX5/C20orf112* fusion genes (Fig. 2*D*).

To confirm cellular localization of PAX5-fusion proteins, we transfected vectors encoding wild-type *PAX5* and *PAX5* fusion genes (*PAX5-ETV6*, *PAX5-FOXP1*, *PAX5-C20ORF112S*, and *PAX5-C20ORF112L*) into 293T cells, fractionated the cytoplasmic and nuclear proteins, and examined the wild-type PAX5 and PAX5-fusion proteins by Western blot analysis (Fig. 2*E*). PAX5-ETV6 protein was detected in both the cytoplasm and nucleus; PAX5-FOXP1 and PAX5-C20ORF112L proteins were predom-

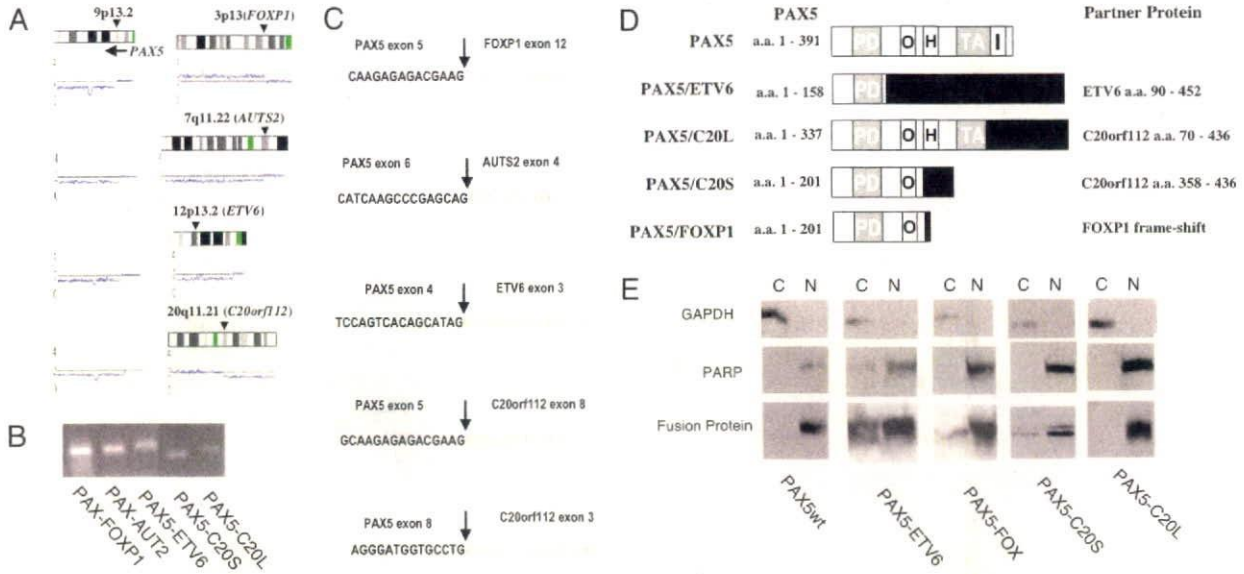

Fig. 2. *PAX5* gene is fused to partner genes. (*A*) Start sites of deletion at 9p13.2 involving the PAX5 gene. (*Left*) SNP-chip data of representative cases with 9p13.2 deletions. A vertical arrow indicates the start sites of 9p deletion that involves the *PAX5* gene. A horizontal arrow shows the direction of transcription of the *PAX5* gene. (*Right*) Chromosomal abnormalities of partner chromosomes. Arrow heads indicate the start sites of duplication or deletions. Genes involved in the start sites are shown. (*B*) Result of RT-PCR. The ALL samples suggesting the presence of PAX5 fusion genes by SNP-chip analysis were examined by RT-PCR using the primers of PAX5 and the respective partner genes. (*C*) Fusion sequences of the *PAX5* and partner genes. Joining sequences of fused transcripts are shown from the indicated exon of the fused gene. (*D*) Schematic structure of wild-type and mutant PAX5. Amino acid positions (aa) of each protein are indicated. PAX5/FOXP1 fusion construct has an early termination codon caused by a frame-shift. PD, paired domain; TA, transcription activation domain; O, octapeptide H, homeodomain-like; I, inhibitory domain. (*E*) Subcellular fractionation of PAX5-fusion proteins. pcDNA vector encoding wild-type PAX5, PAX5-ETV6, PAX5-FOXP1, PAX5-C20ORF112S, or PAX5-C20ORF112L was transfected into 293T cells. Nuclear and cytoplasmic proteins were separated and electrophoresed in the gel. Localization of PAX5-fusion proteins was examined by PAX5 N-terminal specific antibody. Purity of cytoplasmic protein was examined with anti-GAPDH antibody and purity of nuclear proteins with the anti-PARP antibody. C, cytoplasmic fraction; N, nuclear fraction.

inantly localized in the nucleus; and 20% and 80% of PAX5-C20ORF112S proteins were localized in the cytoplasm and the nucleus, respectively (Fig. 2*E*). Localization of the fusion proteins was also confirmed by immunohistochemical staining (data not shown).

Because PAX5-fusion proteins were localized in the nucleus, we analyzed DNA binding affinity of these PAX5-fusion proteins *in vitro*. DNA binding affinity of the PAX5 wild-type and fusion proteins expressed in 293T cells was analyzed by electrophoretic mobility shift assay (EMSA), and signals of probes bound to the proteins were plotted graphically (Fig. 3*A*). Binding activity of each protein in the absence of cold competitor oligonucleotide probe was regarded as 1.0, and the binding activity in the presence of cold competitor oligonucleotide probes was measured. All PAX5-fusion proteins showed similar binding activity to the PAX5 recognition sequences as the wild-type PAX5 (Fig. 3*A*).

***PAX5* Fusion Products Suppressed Transcriptional Activity of Wild-Type PAX5 in a Dominant Negative Fashion, Leading to Inhibition of B-Cell Development.** To examine the effect of PAX5-fusion proteins on transcriptional activity of wild-type PAX5, we performed a reporter gene assay using 293T cells. Cotransfection reporter gene assays using wild-type and fusion *PAX5* expression vectors along with a reporter gene driven by the murine CD19 promoter (which contains three repeats of PAX5 binding sequences) showed that the PAX5 fusion products suppressed transcriptional activity of PAX5 in a dominant-negative fashion (Fig. 3*B*). Expression of wild-type PAX5 proteins was minimally affected by coexpression of PAX5-fusion proteins (Fig. 3*C*), suggesting that PAX5-fusion proteins competed with wild-type PAX5 for the PAX5 binding sequences on the reporter gene.

Further, we transfected vectors encoding either PAX5-C20orf112S or PAX5-C20orf112L, each coexpressing the GFP marker, into Nalm 6 cells (a human B cell ALL cell line, which expresses endogenous PAX5) (data not shown). After transfection, GFP-positive cells were sorted by FACS and expression of PAX5-downstream genes was examined by semiquantitative RT-PCR (Fig. 3*D* and data not shown). We examined 10 downstream target genes (seven positively regulated direct target genes and three negatively regulated genes) of PAX5 (10–12) and found that four, including *ATP1B1*, *BLK*, *NEDD5* and *TCF7L2*, were down-regulated by induction of either PAX5-C20orf112S or PAX5-C20orf112L protein. However, expression of other reported PAX5 downstream target genes, including three positively regulated direct target genes (*IRF8*, *BST1*, *CD19*) and three negatively regulated genes (*CCR2*, *CCR5*, *NOTCH1*) were not affected by the induction of expression of the fusion proteins in these cells.

To examine the effect of PAX5 fusion protein on binding of wild-type PAX5 to the direct target gene *BLK* in the leukemic cells, we performed chromatin-immunoprecipitation (ChIP) assay using Nalm 6 cells transfected with either an empty vector or a construct encoding PAX5-C20orf112S. We used a PAX5 antibody detecting the C-terminal region of the protein, which could detect wild-type PAX5, but not PAX5-C20orf112S, as the C-terminal end of PAX5 was replaced by C20orf112S in this fusion protein. Although wild-type specific PAX5 antibody precipitated the promoter region of *BLK* after transfection of the empty vector, the amount of DNA of the *BLK* promoter region bound to wild-type PAX5 was reduced after transfection of the PAX5-C20orf112S gene (Fig. 3 *E* and *F*).

To examine the effect of PAX5-fusion proteins on B cell development in murine hematopoietic cells, we infected murine

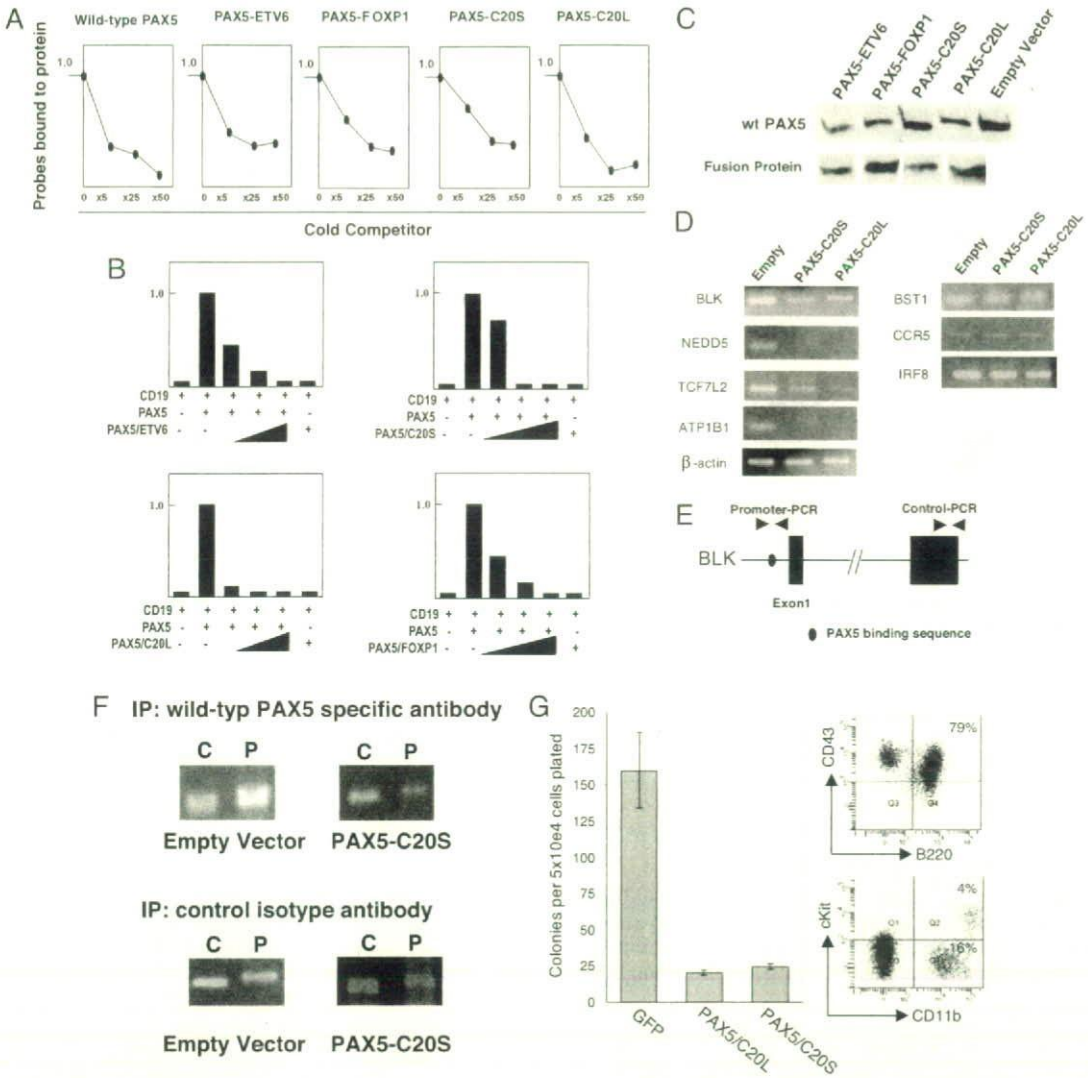

Fig. 3. *PAX5*-fusion proteins suppress transcriptional activity of PAX5 in a dominant-negative fashion and block the growth of B cells. (*A*) Result of EMSA: Wild-type PAX5 and PAX5 fusion were expressed in 293T cells, and nuclear proteins were purified. The purified nuclear proteins were mixed with radioisotope labeled double-strand oligonucleotide DNA, in either the presence or absence of cold competitor oligonucleotides (5-, 25-, and 50-fold cold competitor probes). Intensity of each shifted band indicating DNA–protein complex was measured and plotted graphically. Intensity of shifted bands in the absence of cold competitor probes was regarded as 1.0. (*B*) Reporter gene assay. Wild-type and mutant PAX5 were mixed at a various ratios (1:0, 1:0.3, 1:1, 1:3, respectively, 1 = 500 ng of construct) and transfected. Forty-eight hours later, relative activity of firefly luciferase was measured and plotted. Results represent the mean values of the three experiments. CD19, PAX5 luciferase reporter construct having PAX5 binding region of CD19 promoter; PAX5, wild-type PAX5; PAX5/ETV6, PAX5/ETV6 fusion; PAX5/C20L, long form of PAX5/C20orf112 fusion in which PAX5 exon 8 is fused to C20orf112 exon 3; PAX5/C20S, short form of PAX5/C20orf112 fusion in which PAX5 exon 5 is fused to C20orf112 exon 8; PAX5/FOXP1, PAX5/FOXP1 fusion with an early termination codon caused by a frame-shift after the site of fusion. (*C*) Results of expression of wild-type PAX5 and PAX5-fusion proteins. After cotransfection of equal amounts of vector encoding either wild-type or fusion PAX5 genes into 293T cells, the expression of respective proteins was examined by Western blot. Levels of expression of wild-type PAX5 protein were minimally affected by coexpression of the PAX5-fusion proteins. (*D*) Semiquantitative RT-PCR of downstream target genes of PAX5. Expression of PAX5 downstream target genes was examined by semiquantitative RT-PCR. Nalm 6, a human B cell ALL cell line expressing endogenous PAX5, was transfected with pMSCV-GFP (Empty), pMSCV-GFP-PAX5-C20orf112S (PAX5-C20S), or pMSCV-GFP-PAX5-C20orf112L (PAX5-C20L). GFP-positive cells were sorted and subject to semiquantitative RT-PCR. Optimal cycle numbers to semiquantify the expression of respective genes are as follows; BLK: 25 cycles; Nedd5; 25 cycles; TCF7L2: 25 cycles; ATP1B1: 25 cycles; β-actin: 22 cycles; CCR2: 25 cycles; CCR8: 30 cycles; IRF8: 30 cycles. (*E*) Structure of human *BLK* gene. Structure of *BLK* and primers used for ChIP assay within the 5′ regulatory region (Promoter-PCR) and 3′ end (Control-PCR) of the *BLK* gene is schematically shown. PAX5 binding site in the promoter region is indicated. (*F*) ChIP analysis of the PAX5 binding site in the *BLK* gene promoter. pMSCV-GFP (empty vector) or pMSCV-GFP-PAX5-C20S (PAX5-C20S) was transfected into human Nalm 6 B cell leukemia cells expressing endogenous PAX5. GFP-positive cells were subject to ChIP assay. The cells were fixed in formaldehyde solution and sonicated by ultrasound. DNA-protein complex was incubated with wild-type PAX5 specific antibody, which detected the C-terminal region of PAX5 but not the PAX5-C20orf112S protein (*Upper*). As a control, the DNA-protein complex was reacted with isotype nonspecific antibody (*Lower*). Immnoprecipitated DNA was subjected to PCR to amplify either the *BLK* promoter region containing PAX5 binding sequence (P) or, as an internal control, the 3′ end of the *BLK* gene (C). (*G*) Retrovirus infection experiments. Murine bone marrow cells were collected at 5 days after injection of 5FU. The hematopoietic cells were infected by retrovirus containing pMSCV-GFP empty vector (GFP), pMSCV-GFP-C20orf112L (PAX5/C20L), or pMSCV-GFP-C20orf112S (PAX5/C20S). GFP-positive murine hematopoietic cells were sorted and plated at 5×10^4 cells per plate in methylcellulose containing mSCF, mIL7, and hFL. At 8 days after the plating, the colony numbers were counted (*Left*; results represent means and SD of three experiments). Cell surface antigens on the GFP-positive cells infected with pMSCV-GFP (GFP) at Day 11 were examined by FACS using antibodies against CD43 and B220 (*Upper Right*), c-kit and CD11b (*Lower Right*) antibodies, to confirm the development of B cells.

hematopoietic cells from the bone marrow with retroviral vectors encoding either PAX5-C20orf112S or PAX5-C20orf112L. GFP-positive infected bone marrow cells were sorted by FACS and plated in media containing cytokines that are know to stimulate B cell differentiation (Fig. 3*F*). Murine hematopoietic cells infected with the empty vector showed abundant colonies (Fig. 3*G Left*), and 79% of the cells were B220 positive B cells (Fig. 3*G Upper Right*). In contrast, murine hematopoietic cells infected with either PAX5-C20orf112S or PAX5-C20orf112L formed very few colonies (Fig. 3*G Left*). Most of these colonies were GFP-negative (data not shown), suggesting that these PAX5-fusion proteins impaired B cell development from murine hematopoietic cells.

Discussion

In this study, we describe a paradigm for discovering fusion genes in malignancy by taking advantage of samples with unbalanced translocations and using high density SNP-chip analysis. This technique allows us to identify genes involved in translocations even if chromosomal analysis is not available, especially in solid tumors.

Steps to identify novel fusion genes using SNP chip analysis include (*i*) identify either a deletion or duplication that occurs within two genes; (*ii*) determine whether transcription of both genes is in the same direction; (*iii*) take advantage of ancillary tests such as standard chromosomal analysis or spectral karyotyping (14), which can grossly show that two chromosomes are fused; and (*iv*) design primers of candidate genes and perform RT-PCR to clone fusion genes. Rapid amplication of cDNA ends (RACE) (15) or long-distance PCR (12) also help the cloning of genes involved in translocations. In our SNP-chip data, a number of regions of segmental deletions or duplications were detected (9). Although some of them are simple deletions or duplications at the original sites of the chromosomes, the others are deletions that occurred during chromosomal translocations or when duplicated fragments were inserted into chromosomal sites other than the original region (data not shown). Therefore, data of chromosomal analysis help to define translocations, leading to identification of candidate genes in novel fusion genes.

Recently, Tomlins *et al.* found the fusion genes *TMPRSS2/ERG* and *TMPRSS2/ETV1* in prostate cancers by using expression microarray data (16). They focused on the genes *ERG* and *ETV1*, which are highly expressed in this cancer and examined levels of individual exons of these two genes (16). They found differences in expression of 5′ and 3′ regions of the genes, suggesting that these genes are fused to each other (16). In these fusion genes, the 5′ regions were replaced by the *TMPRSS2* gene, resulting in the differences in the expression of the 5′ and 3′ region of the *ERG/ETV1* genes (16). They also used SNP-chip analysis to identify these fusion genes and found a deletion of a genomic region between TMPRSS2 (21q22.3) and ERG (21q22.2), leading to fusion of these two genes (17). These new technologies, based on oligonucleotide microarrays and bioinformatics, will help to identify fusion genes in cancers.

Our study found that the *PAX5* gene was frequently fused to one of a variety of partner genes. PAX5 is a key transcription factor in the development of B cells (18, 19). We found that these PAX5 fusion proteins suppressed the function of wild-type PAX5 in a dominant-negative fashion and suppressed expression of downstream target genes of wild-type PAX5 in leukemic cells.

We found that when *PAX5* was joined to one of its fusion partner genes, its C-terminal end was replaced by one of the partner genes. Elimination of the C-terminal end of *PAX5* may play an important role in generation of a dominant negative form of mutated *PAX5*. In *in vitro* assays, PAX5-fusion proteins showed a similar affinity as wild-type PAX5 for the PAX5 recognition sequences. Although expression of several downstream targets of wild-type PAX5 was repressed by expression of PAX5-fusion proteins, others were not affected. Binding of transcription factors to DNA can be modulated by cofactors and/or neighboring transcription factors (20). Compared to PAX5, PAX5-fusion proteins may bind more strongly to some target genes and more weakly to others, depending on the contextual environment of the target genes.

Further, our data showed that PAX5-fusion protein inhibited B cell development of hematopoietic cells in a colony formation assay. This result may suggest that PAX5 fusion protein blocked differentiation of hematopoietic cells into mature B cells. PAX5-deficient mice have impairment of B cell differentiation (18). These data suggest that PAX5-fusion proteins may contribute to leukomegenesis by blocking B cell differentiation. It has been suggested that two distinct genetic abnormalities contribute to leukemogenesis in acute myelogenous leukemia (AML); one is mutations promoting cellular proliferation, for example *FLT3* or *RAS* mutations, and the other is mutations blocking differentiation, for example *PML-RARA* or *RUNX1-ETO* (21, 22). PAX5-fusion proteins may cooperate with unidentified mutations promoting cellular proliferation in the ALL cells.

Recently, Mullighan *et al.* have analyzed pediatric ALL samples by high density SNP-chips and found frequent abnormalities of *PAX5* gene (23). Their data also showed that PAX5 fusion products suppressed transcriptional activity of PAX5 in a dominant-negative fashion (23). In addition, other researcher have reported *PAX5* fusion genes, including *PAX5* fused to *ETV6* (12p13) (23, 24), *FOXP1* (3p14) (23), *ZNF521* (18q11) (23), *ELN* (7q11.23) (25), and *PML* (15q24) (26). We have found *PAX5* fused to either *ETV6*, *FOXP1*, *C20orf112* (20q11), or *AUTS2* (7q11.22).

In our study, the function of PAX5 was attenuated by the dominant-negative forms of the fusion products in B cell lineage ALL, suggesting that PAX5 behaves as a tumor suppressor in early B cells, and that impairment of its function can be associated with the development of ALL. In contrast, translocation of the *PAX5* gene to the enhancer region of the Ig heavy chain gene [t(9;14)(p13.2;q32)] or point mutations of the 5′ regulatory region of the *PAX5* gene leads to its overexpression, which is associated with B cell lineage lymphomas (27–29). Also, experimental overexpression of wild-type PAX5 can transform lymphocytes (30, 31). Therefore, an aberrant PAX5 may behave in a dominant-negative fashion at the pre-B stage of B cell development, resulting in ALL; its forced expression in a more mature B cell can lead to lymphoma. Our study showed that PAX5-fusion proteins blocked differentiation of B cells but did not transform them. B cells at different stages of differentiation may need alteration of distinct sets of pathways to transform. Why PAX5 can act as a tumor suppressor in ALL and as an oncoprotein in lymphoma is unclear. Further studies are needed to clarify the mechanism of this paradoxical phenomenon in carcinogenesis.

In summary, we identified multiple fusion genes in ALL by SNP-chip analysis, leading to the exploration of a B cell differentiation block as a contributing factor to the development of ALL. This methodology should help researchers to identify oncogenic fusion genes and explore the mechanism of tumorigenesis in other types of cancers as well.

Materials and Methods

Samples and DNA/RNA Preparation. SNP-chip was performed on 399 pediatric ALL patients consecutively enrolled in the ALL-BFM 2000 trial of the Berlin-Frankfurt-Münster (BFM) study at diagnosis and during remission (350 cases were B cell lineage ALL and 49 cases were T cell lineage ALL) (9). Detailed results of the SNP-chip analysis are published separately (9). The ALL-BFM 2000 study was approved by the local ethics committee. DNA and RNA were extracted from the ALL samples and cell lines by using standard techniques (32). Nalm 6, a human pre-B ALL cell line, was generously provided by Dr. G. Crook (Los Angeles Children's Hospital, Los Angeles, CA) and maintained in RPMI medium 1640 with 10% FBS.

SNP-Chip Analysis. SNP-chip of GeneChip Human mapping 50k array XbaI 240 and/or 250k Nsp were used for this study (Affymetrix Japan). Preparation of samples was reported previously (4, 5). The data were analyzed by CNAG program as previously described (4, 5). All 399 ALL samples and their matched control samples were analyzed by using 50K-SNP chip; selected cases with genomic abnormalities were also analyzed by using 250K SNP-chip.

RT-PCR. RT-PCR was performed by using ThermoScript RT-PCR Systems (Invitrogen) according to the manufacturer's protocol. The primers used for detection of *PAX5* fusion transcripts are listed in Table S2. Expression of PAX5 downstream target genes in Nalm 6 cells after transfection was examined by semiquantitative RT-PCR. The gene names and their primer sequences are listed in Table S3.

Reporter Gene Constructs and Expression Vectors. The PAX5 reporter gene construct with the luciferase gene and PAX5 binding region of the CD19 promoter, as well as the human PAX5 cDNA constructs, were kindly provided by Dr. M. Busslinger (Research Institute of Molecular Pathology, Vienna, Austria). PAX5-fusion constructs were generated by using PCR. All coding regions were ligated into the pcDNA3.1 vectors (Stratagene). Wild-type PAX5 cDNA was ligated into pMSCV vector (Clontech), and EGFP cDNA was ligated under the control of pGK promoter as a marker (pMSCV-GFP-wtPAX5). PAX5-C20orf112S and PAX5-C20orf112L cDNA sequences were also ligated into pMSCV-GFP vectors.

Transfection and Reporter Gene Assay. For reporter gene assays, pMSCV-GFP-wtPAX5 and pcDNA vectors encoding PAX5-fusion genes were cotransfected with the PAX5 reporter construct and pRL (*Renilla* luciferase) vector into 293T cells by using the Effecten transfection kit (Qiagen). Firefly and *Renilla* luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega). Transfection into Nalm6 human pre-B cell ALL cell line was performed with Amaxa nucleofector. GFP-positive cells were sorted by using the MoFlo cell sorter (Dako). Detailed information about the procedure is described in *SI Text*.

Retrovirus Transduction into Murine Hematopietic Cells. Retrovirus containing pMSCV-GFP (empty), pMSCV-GFP-PAX5-C20orf112S, and pMSCV-GFP-PAX5-C20orf112L was generated. The retrovirus was transfected into murine bone marrow cells as previously reported (33). After the transfection, GFP-positive cells were sorted and plated into methylcellulose cultures (M3231; Stem Cell Technologies) as previously described (33). Surface antigens (CD43, B220, c-kit, and CD11b) of these GFP-positive cells were examined by using FACScan (Becton–Dickinson). Detailed information of the procedure is described in the *SI Text*.

Subcellular Fractionation of Proteins and EMSA. Forty-eight hours after transfection of vectors into 293T cells, the cells were subjected to subcellular fractionation with the CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich). Detailed information of the procedure is described in the *SI Text*.

Purified nuclear proteins from the cells were also subjected to EMSA as previously reported (34). Detailed information of the procedure is described in the *SI Text*.

Chromatin Immunoprecipitation (ChIP) Assay. ChIP assay was performed with the Magna ChIP A kit from Millipore according to the manufacturer's protocol. pMSCV-GFP or pMSCV-GFP-C20orf112S were transfected into Nalm 6 cells as described above, and GFP-positive cells were sorted by MoFlo (Dako). Precipitated DNA was recovered and subjected to PCR to amplify the *BLK* promoter region and the 3′ end of the *BLK* gene (internal control). The primer sequences used for ChIP assay are listed in Table S4. Detailed information of the procedure is described in the *SI Text*.

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Invited Review

Current Risks in Blood Transfusion in Japan

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CONTENTS:

SUMMARY: Over the past decades, the incidence of transfusion-transmitted diseases has been dramatically reduced. These reductions have been due to a multifocal approach to the collection, processing, and release of blood components. The estimated risks of transfusion-transmitted hepatitis viruses are now extremely small, but the possibility of infections with emerging pathogens always exists because preventive measures may not be available for all cases. Thus, some patients may be harmed before preventive measures are introduced. Beside transfusion-transmitted infections (TTI), unsolved residual risks such as transfusion-related acute lung injury or incompatible blood components transfusion still exist as major concerns. Continuous efforts toward research on and the prevention of adverse reaction-related blood components must be made to ensure blood safety. The purpose of this article is to introduce the concept of the current risks of transfusion including TTI, review the preventive measures already implemented, and discuss future visions for transfusion safety in Japan.

1. Introduction

Transfusion safety is of the utmost concern, and much effort has been expended on measures to reduce the risk of transfusion-transmitted infectious agents. Since the onset of the human immunodeficiency virus (HIV) epidemic, effective screening tests have been implemented. Moreover, a multifocal approach to the collection, processing, and release of blood components has been added, and as a result remarkable improvements have been made in blood safety. However, the current strategy could not eliminate all transfusion-transmitted infectious agents, i.e., not only known pathogens but also unknown new agents, and some patients may be harmed before preventive measures are introduced. In Japan, the Japanese Red Cross (JRC), the sole provider of labile blood components in Japan, is responsible for blood products in accordance with the Pharmaceutical Affairs Law and has made much effort to improve blood safety.

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Blood donors should answer many questions about their medical history and their risk factors. Their blood samples should be screened for indicators of infections such as syphilis, parvovirus B19, hepatitis B virus (HBV), hepatitis C virus (HCV), HIV types 1 and 2, and human T-cell lymphotropic virus (HTLV) types I and II. Blood is further tested for cytomegalovirus (CMV) antibody (Ab) before transfusion into patients who are at high risk for CMV disease. Similarly, hepatitis E virus (HEV) is screened as a trial in the Hokkaido district. Even though multifocal approaches to blood safety have been introduced in Japan, unresolved residual risks still exist. These include not only transfusion-transmitted infections (TTI) but also immunological adverse reactions such as transfusion-related acute lung injury (TRALI) and some allergic reactions. For the time being, transfusion components are derived from human blood; therefore, a "zero risk" blood transfusion is never possible. However, it is clear that the application of safety measures and a credible surveillance system which identifies the current transfusion risks will enable transfusion therapy to be safe. A continuous effort toward the research and prevention of adverse reaction-related blood components should be made.

The purpose of this article is to demonstrate the current transfusion risks, describe various approaches that have been implemented for blood safety, and discuss future visions about transfusion medicine in Japan.

2. History of transfusion medicine in Japan

The first transfusion experience in Japan was reported in 1918. Since then, most of the blood components derived from sold blood have been transfused. In 1964, a law came into effect and new JRC statutes were established. At the same time, the Cabinet made a decision that all blood components should be derived from donations, instead of blood sales, and all blood components have been supplied through donated blood since 1969. In 1972, the screening of hepatitis B surface antigen (HBsAg) was begun. The HTLV-I Ab test was added in 1986. In the same year, JRC also began to screen for HIV Abs as a measure to avoid an HIV epidemic.

In 1989, screening strategies for hepatitis B core antibody (HBcAb) and HCVAb were added. JRC established a hemovigilance system in their society and began to collect information on transfusion-related complications including TTI on a voluntary basis in 1993. As a part of the look-back system, JRC began to store 6-ml frozen repository samples from all blood donations in 1996 (1). Since 1999, nucleic acid amplification tests (NAT) for HBV, HCV, and HIV for labile blood products were introduced, and the pool size of NAT was reduced from 500 to 50 the following year (2,3). Since 2004, the pool size of NAT has been reduced from 50 to 20.

In order to investigate the causal relationship between blood components and incidents after transfusion, a look-back system regarding TTI was started nationwide in 2003, and the following year, 6-month-quarantine storage for fresh frozen plasma (FFP) was achieved. In 2005, pre-storage leukocyte-reduction for apheresis-derived platelets was started, and 2 years later, this approach was adapted for all labile blood components. In order to reduce the risk of bacterial contamination, diversion of initial blood flow was adopted in 2006.

Since 2005, people with a history of travel to some European countries, especially England, where bovine spongiform encephalopathy (BSE) is epidemic have been rejected as blood donors.

As noted above, more and more new technologies and/or additional interventions have been adopted over time to achieve the goal of "zero risk."

3. Current risks in transfusion

3-1. Transfusion-transmitted infectious diseases

3-1-1. HBV

Repository samples from all donors, which have been stored since 1996, made it easy to analyze the causal relationship between blood components and recipients. At present, approximately more than 10 cases per year are reported (4) in spite of various approaches to prevent transmission. The NAT-window period (5,6) and low titer of HBV DNA, which cannot be detected in occult HBV-carrier donors, are considered to be the main reasons for this (7-12). A JRC look-back survey reported that the risk of HBV infection caused by blood components from occult HBV carriers with low anti-HBc titers is more than 10-fold lower than the risk caused by donors in the NAT-window periods (6).

According to the JRC reports, it is estimated that the risks of HBV transmission range from 1 in 340,000 bags to 1 in 450,000 bags in Japan (13,14). Satake et al. estimated that the total number of HBV-TTI cases is 17 to 20 per year (1/0.27-0.32 million donations) in Japan out of 5.4 million annual blood donations. This implies that approximately 85% of the HBV infections are caused by donors in the NAT-window period (6).

3-1-2. HCV

Before the implementation of NAT screening, many suspected cases were reported every year. However, during the past few years, one case has been reported every year. The current rate of post-transfusion HCV infection from the donor is estimated to be 1 in 22 million donors, which corresponds to the risk of screening a test-negative, -individual-NAT-positive blood transfusion (13,14).

3-1-3. HIV

One case of HIV transmission due to whole blood transfusion was reported in 1997. Two cases of infected FFP and erythrocytes from the same donor also were reported in 1999. After the implementation of NAT, only one case of FFP-related HIV was reported in 2003. Therefore the risk of transmission is very low, estimated to be 1 in 11 million donors (13,14). However, the number of HIV-infected people in the population has been increasing in Japan. Also, the number of blood donors in which HIV is detected positively has been increasing gradually and finally exceeded 100 people per year in 2007 (Fig. 1) (15).

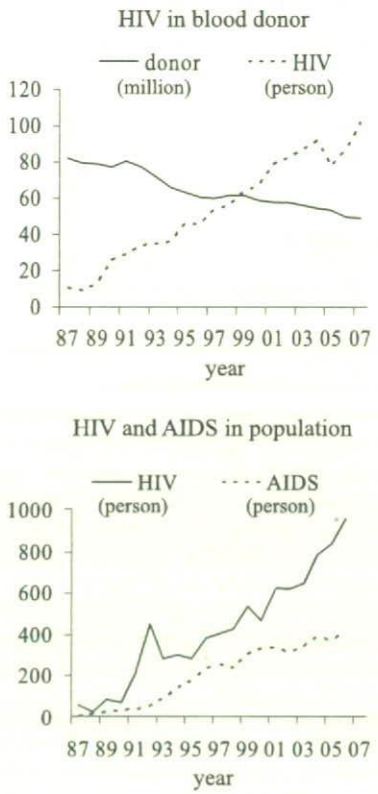

Fig. 1. Trends of the HIV infected rate in population and donation in Japan.

3-1-4. HTLV-I

HTLV-I, the first human retrovirus discovered, is well known as an etiologic pathogen of adult T-cell leukemia/lymphoma (ATL) and other associated diseases. It has been shown to have high seroprevalence in some endemic areas, especially Southwest Japan, the Caribbean islands, and parts of Africa. Its major routes of transmission are considered to be blood transfusion, breast milk feeding and sexual contact (16,17).

Table 1. Previously reported cases of confirmed bacterial contaminated blood components in Japan

Year	Detected bacteria species		Caused blood component
	Patient	Blood component	
1993	*Bacillus subtilis*	NT	RC-MAP
1994	*Serratia marcescens*	NT	PC
1995	*Acinetobacter calcoaceticus*	NT	RC-MAP
1996	*Staphylococcus aureus*	NT	PC
1996	G(+) rod	NT	RC-MAP
1998	*Morganella morganii*	NT	PC
2000	*Bacillus cereus*	*Bacillus cereus*	RC-MAP
2000	*Streptococcus pneumoniae*	*Streptococcus pneumoniae*	PC
2003	*Yersinia enterocolitica*	*Yersinia enterocolitica*	RC-MAP
2006	*Yersinia enterocolitica*	*Yersinia enterocolitica*	RC-MAP
2006	*Yersinia enterocolitica*	*Yersinia enterocolitica*	RC-MAP
2006	*Staphylococcus aureus*	*Staphylococcus aureus*	PC

RC-MAP, erythrocyte concentrate in mannitol-adenine-phosphate solution; PC, platelet concentrates; NT, not tested.

Therefore, preventive measures involving particle agglutination (PA) were implemented in 1986. Moreover, a second generation of PA methods was released for donor screening. Inaba et al. evaluated the efficacy of this screening and HTLV-I prevalence in blood donors after screening estimated the prevalence to be 1 in 45,560 (0.0022%) (18). No confirmed case transmitted by blood components has been reported to the JRC; however, its long latent period and transmission routes other than transfusion make the prevention rate uncertain.

3-1-5. Bacteria

Transfusion-transmitted bacterial contamination of platelets is the most common cause of fatality-related blood components, because the storage of platelets at room temperature to maintain its function is also suitable for bacterial growth. Therefore, numerous countries have introduced culturing-based screening methods to detect bacterial-contaminated platelets. However, after the implementation of these methods, death from bacterial sepsis has continued to be reported because erythrocytes are not screened for bacteria, and current screening methods based on culturing are not entirely satisfactory.

In the United States (US), before the implementation of culturing methods, an average of 11.7 deaths from sepsis per year were reported, whereas 7.5 per year were reported after these detection methods were introduced (19). According to the 6 years' experience of using the BacT/ALERT system in the US, between 0.03 and 0.12% of platelet concentrates (PCs) with a negative culturing test result were still contaminated with bacteria, i.e., false negatives were reported (20).

In Japan, screening methods for platelets have not yet been introduced. We evaluated the efficacy of DOX™ (Daikin Industries, Osaka, Japan), a commercially available system which has been developed to detect contaminated food by measuring the oxygen potential for contaminated PCs. Six species were inoculated into PC, and their dissolved oxygen potentials were measured consecutively (21,22). As a result, this system detected aerobic bacteria in PC within 20 h if their initial concentration was more than 10^3 CFU/ml.

Fatalities from bacterial sepsis are extremely rare and have been reported once every few years (23). However, we have experienced two fatalities from bacterial-contaminated platelet recently. One case was reported in 2000, caused by *Streptococcus pneumoniae* (24), and another case occurred in 2003, caused by a *Staphylococcus aureus*-contaminated platelet (25). In both cases, the patients suffered from malignant hematological diseases. Reported cases of bacterial contamination in Japan are described in Table 1. Since 2007, pre-storage leukocyte-reduction procedure and diversion of initial blood flow have been introduced in Japan. According to the JRC's report, nearly 6,000 blood aliquots from whole blood collected by either the conventional method or from the initial drawn blood flow were cultured using an automated culture system. As a result, the detected rate of bacterial contamination was remarkably reduced from 7 of 2,967 samples (0.24%) to 2 of 2,890 samples (0.07%) after implementation of the diversion (26).

National Blood Service (NBS) in the United Kingdom (UK) also reported that diversion together with improved donor arm disinfection has improved the reduction rate in contamination from 47 to 77% (27).

3-1-6. Prion and other emerging pathogens

Variant Creutzfeldt-Jacob disease (vCJD) was first identified in 1996 in the UK (28,29), and it is considered to be the result of human exposure to the BSE agent. Since then, vCJD patients have been identified in many European countries, especially in the UK. In 2004, the reports showed that vCJD can be transmitted by blood transfusions (30,31). The strategy for preventing trasmission through transfusion has been difficult because there is no effective screening method to determine if a blood donor is infected, and this disease has a long incubation period. Therefore, patients probably received blood products from donors who were asymptomatic at the time of donation. The US instituted a policy in which donations from people who spent at least 6 months in certain western European countries or 3 months in the UK between 1980 and 1996 were excluded. A similar policy has been applied to potential donors in many countries. In Japan, people who spent even one day in the UK from 1980 to 1996 and cumulative periods of 6 months in western European countries where BSE is epidemic were rejected as blood donors.

Consequently, donor deferral was roughly o 6% as a result of this policy. Recently, a number of companies have been developing prion removal filters. Asahi Kasei Medical Co., Ltd. (Tokyo, Japan) has developed an integrated filter which has the functions of prion removal and leukocyte reduction (32,33).

Pall Co., Ltd. (East Hills, N.Y., USA) gained a Council of

Europe (CE) mark for their device "Pall Leukotrap Affinity Prion Reduction Filter (LAPRF)," a new leukocyte reduction filter for the removal of infectious prion from erythrocyte concentrates in 2005 (34,35). Pathogen Removal and Diagnostic Technologies, Inc. (PRDT), which is a joint venture company of the American Red Cross and ProMetic BioSciences, established "P-Capt," which has high prion-binding affinity and also received CE mark in 2006, in cooperation with Macopharma (36).

Some pathogen agents carried by mosquitoes, such as chikungunya virus in the Indian Ocean, West Nile virus in the US, and malaria are widely known as transmitted infectious pathogens (37). Fortunately, this is not an issue of concerne in Japan at present, but potential donors move frequently throughout the world, and some materials imported from abroad may carry mosquitoes. We are collecting information carefully, and we have to manage them in the near future.

Similarly, HEV has been considered to be an imported infectious disease from its epidemic area in the developed countries. However, the epidemiologic study revealed that 2-14% of healthy populations were anti-HEV IgG positive (38), and approximately 13% of the non-A, -B, and -C acute hepatitis cases in Japan were caused by HEV (39). Moreover, the discovery in 2001 of an indigenous Japanese strain of HEV, JRA1, from a patient who had never been abroad, had a great impact on blood safety in our country (40,41). Under these circumstances, HEV screening using a real-time reverse transcription (RT)-polymerase chain reaction (PCR) system has continued as a trial in the Hokkaido district, northern part of Japan.

Blood is also tested for CMV Ab and provided to patients who are at an increased risk for CMV disease in Japan.

3-2. Non-infectious reactions

3-2-1. Hemolytic reactions

Hemolytic reactions are classified into acute hemolytic reactions and delayed hemolytic reactions. Most important hemolytic reactions involve incorrect blood components (IBCT). IBCT has rarely been reported to JRC as an adverse reaction, because it is regarded as a transfusion error. The surveillance of ABO-incompatible blood transfusions was conducted based on an anonymous questionnaire by the Japanese Society of Blood Transfusion for 5 years from 2000 to the end of 2004 (42). This surveillance targeted 1,355 hospitals in Japan, and data were obtained from 829 hospitals among them (61.2%). According to the data, 60 cases of ABO-incompatible transfusion were reported, and 31 of them involved erythrocyte concentrates. Of 31 cases, 22 were due to major mismatches, and others were due to minor mismatches. The current incidents collection system used by JRC is based on voluntary reporting; therefore, the number of reported IBCT cases might be underestimated.

3-2-2. Non-hemolytic reaction

Minor allergic reactions such as urticaria, fever, and dyspnea make up a major portion of non-hemolytic reactions. These include transfusion-associated graft versus host diseases (TA-GVHD) and TRALI.

3-2-3. TA-GVHD

Once TA-GVHD occurs, it is almost always fatal with a very rapid and fulminant course. The mechanism of this condition involves the activation of donor lymphocytes against recipient human leukocyte antigens (HLA). The risk increases in proportion to the degree of HLA haplotype-sharing between donors and patients. In Japan, this condition is a serious problem. Indeed, its incidence is 5-10 times higher than in European countries (43,44). JRC collected information and conducted a national survey in 1991, and a microsatellite DNA assay to identify TA-GVHD has also been developed (45-47). Consequently, JRC has begun the practice of irradiating the blood components supply throughout the country. Since 2000, no confirmed TA-GVHD case has been reported to JRC.

3-2-4. TRALI

TRALI is a serious clinical syndrome involving shortness of breath, hypoxemia and non-cardiogenetic pulmonary edema, associated with HLA/Abs or neutrophil antigens. JRC has gathered information on TRALI since 1997. As knowledge of TRALI has grown, the number of reported TRALI cases has increased. However, the definition of TRALI remains controversial, and it is likely that only a portion of TRALI cases are collected. Other similar serious symptoms which are not included in the definition occur, and treatments have not been developed. Supportive diagnostic evidence includes identifying neutrophil or HLA Abs in the donor or recipient plasma. Among the blood donors, multiparous women frequently have these antibodies. Therefore, in many developed counties, women are not permitted to be plasma donors. In Japan, however, this policy has not been applied.

4. Traceability of causal relationship between blood components and incidents by JRC

JRC has conducted the following tests on residual blood products, plasma derivatives, and recipient blood to identify the causes of adverse reactions and infectious diseases. The contents of the current tests to trace such causes are described in Table 2 (48).

Table 2. Currently conducted tests to identify the causal relationship between blood products and adverse reaction after transfusion according to the classification of reaction type

1. Transmitted infectious diseases
A. Virus
1. Serological test: serological markers related to suspected infections
2. NAT: (1) Detection of suspected viral genome
(2) Evaluation of viral genome sequence homology
B. Bacteria
1. Detection of bacteria by methods based on blood culturing
2. Identification of bacterial species by Gram's stain
3. Detection of endotoxins of Gram-negative bacteria
2. Non-infectious diseases
A. Non-hemolytic adverse reaction
1) Allergic reaction
1. Anti-human leukocyte antigen antibody
2. Anti-platelet antibody
3. Anti-granulocyte antibody
4. Anti-plasma protein antibody: against 6 plasma proteins, including anti-haptoglobin (HP) antibody and anti-immunoglobulin A (IgA) antibody
5. Plasma protein deficiency
2) TA-GVHD
1. Micro-satellite DNA assay
2. Chimerism test on recipient blood
B. Hemolytic adverse reaction
1. Re-check of the blood group and Coombs test
2. Detection of irregular antibody

NAT, nucleic acid amplification tests; TA-GVHD, transfusion-associated graft versus host diseases.

5. Detection strategy versus pathogen reduction for transmitted diseases

At present, detection strategies such as screening tests for known pathogens for which the methods have already been developed are added yearly to maintain the blood components' safety. However, the current strategy does not prevent all of the transfusion-transmitted pathogens. Considering that the use of human blood as a raw biological source is unsafe, screening tests alone cannot exclude all of the potential pathogens. Therefore, we have to consider the introduction of some alternative or additional preventive measures. Some pathogen reduction systems to damage pathogen nucleic acids to proliferate have been developed and some are now under development. Pathogen inactivation (PI) technology using methylene blue plus visible light or solvent-detergent treatment for plasma has been introduced in some European countries and has a track record of more than 10 years (49-51). Similar technologies involving amotosalen (S-59) plus ultraviolet (UV) A light have recently become available for plasma (52). Only amotosalen and riboflavin UV light treatment have obtained the CE mark in Europe, and they have been under evaluation for use with platelets (53,54). With regard to these methods, concern remains regarding cost, process operation changes, ability to inactivate, and ineffectiveness against prions, non-enveloped viruses, spore-formed bacteria and viruses which exist in exceedingly high concentrations in blood. Damage to the products which results in reduction of coagulation factor activities, deterioration of platelets, toxicity, and mutagenicity in recipients is also controversial (55-57). These residual risks are still a major concern to the public, politicians, regulatory agencies, and blood component providers. A recent consensus conference recommended that PI should be implemented when a feasible and safe method to inactivate a broad spectrum of infectious agents is available (58-60).

In Japan, the delegates on behalf of the Japanese Society of Transfusion Medicine and Cell Therapy (JSTMCT) visited some European countries and collected the current information. Additional detection strategies and undeveloped pathogen reduction technology will be extensively debated over the next few years. But it is obvious that TTI is not static and new agents continue to emerge; therefore, we have to carefully watch the circumstances and collect worldwide information.

6. Hemovigilance

Since the AIDS epidemic, developed countries, especially in Europe, took swift action to try to keep records related to transfusion therapy to help ensure blood safety. One method for doing so is called hemovigilance, which is a system for collecting information on unexpected events from donors after drawing blood to the adverse reactions of the patients after transfusion. Various hemovigilance models are usede around the world, depending on social security and national priorities (61-64). JRC has collected transfusion reaction and infectious disease transmission data since 1993, in accordance with the Pharmaceutical Affairs Law. Reporting by medical institutions is voluntary and targets relatively moderate to severe adverse events.

In 2007, JSTMCT established a hemovigilance committee to cooperate with medical institutions and JRC. Seven university hospitals agreed to report all adverse transfusion events

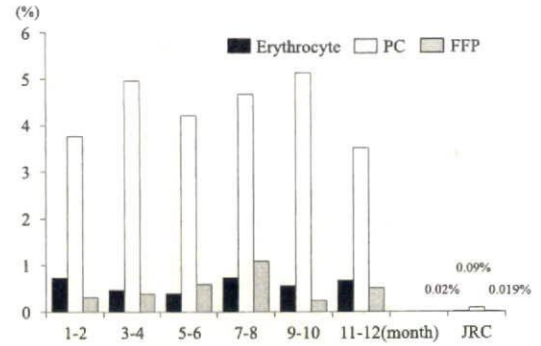

Fig. 2. Bimonthly variability in the reporting rate according to the responsible blood components. PC, platelet concentrates; FFP, fresh frozen plasma.

bimonthly through an anonymous, secure, online portal. Participants also entered the total number of blood products issued over each reporting period. Online access and data entry were made easy, with 16 categories of symptoms and 8 diagnoses. Adverse event rates were calculated automatically and were provided to the participants continuously. As a result of this pilot study, the total number of blood products issued corresponded to about 1% of the total issued in Japan. Six hundred seventy-five transfusion-related adverse events were reported in 2007 by 7 hospitals. Most of them were non-hemolytic transfusion reactions. The reported reaction rates were 0.54 and 0.63% for erythrocytes and plasma, respectively, and 3.4% for PCs in this trial. On the other hand, 0.02% for erythrocytes, 0.018% for plasma, and 0.09% for PCs were nationally reported to JRC (Fig. 2). Hemovigilance such as in this system by a third-party service through an anonymous online portal revealed a high incidence of adverse events, including relatively mild reactions, which physicians previously thought unnecessary, meaningless, or bothersome to report to JRC. Easy online access, anonymity, and the motivation of participating institutions likely contributed to this outcome. This system and the preexisting JRC hemovigilance will complement each other, or rather achieve a better harmonization for future hemovigilance systems (65,66).

7. Conclusion

Current multifocal approaches to blood safety have dramatically reduced the risks related to blood transfusion. However, residual low risks are still a major concern, and we are under pressure to maintain blood product safety. Current approaches have had limited success, and the source of the blood products is raw human blood. In order to improve the safety of blood products, we need to adopt safer alternatives and/or additional preventive measures. Each country has its own circumstances, such as politics, manufacturing, medical resources, and social services, related to transfusion medicine, and each country must develop its most suitable solution.

Consequently, one action taken in one country would not necessarily be an appropriate procedure in another country. It is important to share information and develop standards in transfusion medicine worldwide. However, it is important to remain focused on blood product safety and to track the effectiveness of our policies at all times.

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