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Immunoglobulin Gene Transcripts Have Distinct V_HDJ_H Recombination Characteristics in Human Epithelial Cancer Cells*

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Jie Zheng[‡1], Jing Huang[‡1], Yuntao Mao[‡], Shiqing Liu[‡], Xin Sun[‡], Xiaohui Zhu[‡], Teng Ma[‡], Li Zhang[‡], Jiafu Ji[§], Youhui Zhang[¶], C. Cameron Yin[‖], and Xiaoyan Qiu[‡2]

From the [‡]Peking University Center for Human Disease Genomics, Beijing, 100191, China, the [§]Department of Surgery, Peking University School of Oncology, Beijing, 100142, China, the [¶]Department of Immunology, Cancer Institute, Chinese Academy of Medical Science and Peking Union Medical College, Beijing, 100021, China, and the [‖]Department of Hematopathology, University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

It was well accepted that only B-lymphocytes and plasma cells expressed immunoglobulin (Ig) gene. However, our group and others have confirmed that non-B-cells, such as epithelial cancer cells, can also express Ig. The aim of this work is to elucidate the role of non-B-cell-derived Ig by investigating the characteristics of the Ig heavy chain (IgH) gene repertoire in epithelial cancer cells. We cloned and sequenced 89 V_HDJ_H (V-D-J recombination of the IgH variable region) transcripts by microdissecting cells from eight different types of epithelial cancers and two cancer cell lines (HT-29 and HeLa S3). The cancer-derived Ig gene repertoire showed specific restricted patterns of V_HDJ_H recombination with seven sets of predominant V_HDJ_H sequences. Surprisingly, within a set of V_HDJ_H recombination, the variable (V) sequences derived from different cancer types had not only identical heavy chain variable (VH), diversity (D), and joining (JH) segments usage, but also identical junctions and mutation targets in the V_H region. The $V_{H\gamma}DJ_{H\gamma}$ (but not $V_{H\mu}DJ_{H\mu}$) in the cancer-derived sequences had a high mutation rate; however, it was shown that the mechanism of hypermutation was different from antigen selection in B-cell-derived $V_{H\gamma}DJ_{H\gamma}$sequences. In contrast to $V_{H\mu}DJ_{H\mu}$, the $V_{H\gamma}DJ_{H\gamma}$ sequences did not appear to originate from classical class switching. These results suggest that cancer-derived Ig genes have a distinct repertoire that may have implications for their role in carcinogenesis.

Immunoglobulins (Ig) were discovered more than a century ago, yet the understanding of these proteins continues to evolve. Until 1950, most scientists believed that cells from various types of tissues could express Ig (1). However, it was shown that B-lymphocytes from bone marrow secreted Ig, although other hematopoietic cells did not (2), and that levels of serum Ig decreased with B-cell disfigurement (2, 3). These were thought to indicate that only B-lymphocytes could express Ig; non-immunocytes could not.

In 1976, Tonegawa discovered that Ig gene recombination was the mechanism behind antibody diversity in B-lymphocyte-derived plasma cells. Ig gene recombination, as theorized previously by Dreyer and Bennett, was confirmed to exist in mouse myeloma cells using a probe against the Ig mRNA kappa chain (4, 5).

Subsequently, Cleary *et al.* compared the restriction enzyme map of the Ig gene in B-lymphocytes with that of the genes in cell types such as germ-line using Southern blot analysis and found that B-cell and non-B-cell restriction maps differed. These results further strengthened the hypothesis that Ig gene recombination only occurred in B-lymphocytes. Consequently, Ig gene recombination became a criterion for identifying B-cells (6, 7). Some tumor cells expressing both epithelial cell markers and Ig gene recombination were thus believed to originate from B-cells (6, 8).

Immunoglobulin gene recombination has been detected in T-cell lymphomas and acute non-lymphocytic leukemias (9, 10). However, there is no substantial evidence that Ig gene recombination, transcription, and production could occur in non-immunocytes.

Patients with non-hematopoietic tumors, including carcinomas of the brain, breast, colon, and liver, may have elevated levels of serum IgG, IgA, and/or IgM (11–13). Additionally, many patients with malignant tumors of epithelial origin have been shown to have monoclonal or oligoclonal gamma globulinemia (14–16). These antibodies had been presumed to be produced by B-lymphocytes and plasma cells. However, recent studies from our group and others have demonstrated that both malignant and normal epithelial cells could express Ig.

In 1996, we first reported the detection of IgG-like molecules in breast and colon carcinoma cells and showed that these molecules were not present in their normal epithelial cell counterparts by immunohistochemical staining and Western blot analysis (17). In studies of human cancer cell lines, IgG-like proteins were detected in both the tumor cells and the culture supernatant (18). Kimoto (19) identified transcripts of the Ig constant region and the T-cell receptor (TCR) gene in five epithelial-derived cancer cell lines (SW1116, HEp2, MCF-7, MDA-MB-231, and HC48) using

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[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed: Center for Human Disease Genomics, Peking University, 38 Xue-yuan Road, 100191, Beijing, P. R. China. Tel.: 86-10-82802846-5038; Fax: 86-10-82801149; E-mail: qiuxy@bjmu.edu.cn or xqiu@mdanderson.org.

nested reverse transcription-PCR (RT-PCR).[3] In 2003, we demonstrated that tumor cells isolated from epithelial cancers and cell lines could secrete IgG using Western blot analysis and N terminus sequencing, and we detected both cytoplasmic and secreted IgG in cells from carcinomas of the lung, breast, liver, and colon, as well as epithelial cell lines (20). IgG transcription was also detected by *in situ* hybridization, Northern blot analysis, and single cell RT-PCR (20). In 2004, it was reported that human cervical cancer cells could express Ig mRNA and protein (21). Recent studies have also confirmed the expression of Ig and activation-induced cytidine deaminase (AID) in six breast cancer cell lines (BT474, MDA-MB-231, MCF-7, SKBR3, T47D, and ZR75-1) (22). Furthermore, we recently reported that IgA and IgG were expressed in numerous oral epithelial tumor cells (23). Despite the detection of Ig in numerous cancer cell types, Ig specificity and variable region repertoire are poorly characterized.

B-cells are known to generate Ig diversity by several mechanisms. During the formation of Ig in B-cells from bone marrow, two recombinant events bring different V_H, D_H, and J_H exons together to form heavy chains. Additionally, short sequences are inserted between V_H and D_H and between D_H and J_H to generate further diversity. Subsequent encounters with antigens in the germinal centers drive B-cell to undergo somatic hypermutation (SHM) and class switching, thus generating even greater diversity.

In the present study, we analyzed the V region transcripts in rearranged IgH in eight cancer cell samples from microdissected epithelial cancer tissue and in two cell lines (HT-29 and HeLaS3) using RT-PCR and sequence analysis. We found that cancer-derived Ig genes show classic V_HDJ_H recombination. Reminiscent of classical recombination, additional short sequences were inserted between the V_H and D_H segments and the D_H and J_H segments, Rearranged Ig μ- and γ-chain gene sequences were expressed in cells, and SHM was observed in the V_H segment of the γ-chain genes. However, the cancer-derived Ig gene repertoire also displayed several distinct characteristics.

EXPERIMENTAL PROCEDURES

Sample Assays—For laser capture microdissection (LCM) and RT-PCR analysis of the cancer-derived Ig gene, eight tumor samples from therapeutic excisions of breast invasive ductal carcinoma ($n = 3$), colon carcinoma ($n = 2$), squamous cell carcinoma of the lung ($n = 1$, from the tissue bank of Peking University School of Oncology), squamous cell carcinoma of the oral cavity ($n = 1$), and basal cell carcinoma of the oral cavity ($n = 1$) from the Department of Pathology at Peking University School of Stomatology were included with informed consent from the patients. Ethical approval of the study was granted by the Peking University Health Service Trust Research Ethics Committee. Carcinoma samples were embedded in Tissue-Tek OCT Compound (Sakura, IMEB International Medical Equipment, Inc., San Marcos, CA) and snap-frozen in liquid nitrogen immediately after surgery. Serial frozen sections (8 μm) were cut with a cryostat and mounted on slides treated with 0.1% DEPC for sterilization. Sections were air-dried, fixed in 70% ethanol, and evaluated with hematoxylin and eosin stain or immunohistochemical staining for LCM.

Immunohistochemistry—The slides were then incubated with 0.3% hydrogen peroxide for 5 min, washed with PBS, and blocked in PBS with 10% normal goat serum for 10 min. After removal of excess blocking buffer, indirect immunohistochemical staining was performed with monoclonal antibodies against human epithelial cell adhesion molecules (EpCAM) (1:100, Abcam, Cambridge, MA). Slides were incubated at 37 °C for 45 min, washed thoroughly, and then incubated with horseradish peroxidase-labeled goat anti-mouse IgG (1:100, Dako, Carpinteria, CA) at 37 °C for 45 min. Slides were washed again and bound antibodies were detected using 3,3′-diaminobenzidine (DAB, Sigma Aldrich). The mouse IgG was used as an isotype control.

HT-29 and HeLa S3 Cell Cultures—HT-29 (from colon cancer) and HeLa S3 (from cervical cancer) cell lines were supplied by the Peking University Center for Human Disease Genomics. These two cancer cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone/Thermo Fisher Scientific Inc., Waltham, MA) and L-glutamine (2 mM) at 37 °C in a humidified 5% CO_2.

Isolation and Preparation of Mononuclear Cells from Peripheral Blood—Two samples of peripheral blood were obtained from two healthy donors. Mononuclear cells (MNC) were isolated from 5 ml of peripheral blood using two-step discontinuous Ficoll-Hypaque gradients (Second Chemistry Factory, Shanghai, China). The white gradient layer containing MNC was recovered and washed with 0.01 M PBS, and the isolated MNC used immediately for total RNA extraction.

LCM and RT-PCR of Cancer Cells—LCM was carried out as previously described (20). Briefly, to minimize contamination of infiltrating B-lymphocytes or plasma cells in cancer tissues, only large $EpCAM^+$ cells in cancer cell nests without lymphocyte or plasma cell infiltration were dissected from fresh biopsy tissues of carcinomas of the breast, colon, oral cavity, and lung by LCM. Total RNA of microdissected cancer cells was extracted using RNeasy Micro kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was carried out with the Sensiscript RT kit (Qiagen) according to the manufacturer's instructions. Touchdown PCR was then performed using 1 μl of each reverse transcription reaction with LA Taq polymerase (TaKaRa Bio USA, Madison, WI) as previously described (20). To amplify the human IgVH gene of the γ chain and the μ chain by nested PCR, the first round of PCR was carried out with upstream primers for VH1 (5′-GAGGTGCAGCTCGAGGAGTCTGGG-3′), VH2 (5′-CAGGTGCAGCTCGAGCAGTCTGGG-3′), VH3 (5′-CAGGTACAGCTCGAGCAGTCAGG-3′), and VH4 (5′-CAGGTGCAGCTGCTCGAGTCGGG-3′), coupled with CH1 region primer (CγCH1, 5′-ACACCGTCACCGGTTCGG-3′; CμCH1, 5′-ACGCTGC-

[3] The abbreviations used are: RT-PCR, reverse transcription-PCR; AID, activation-induced cytidine deaminase; PBS, phosphate-buffered saline; SHM, somatic hypermutation; LCM, laser capture microdissection; EpCAM, epithelial cell adhesion molecule; MNC, mononuclear cell; PBL, peripheral blood lymphocytes; CDR, complementary determining region; FWR, framework regions.

TCGTATCCGACGGG-3′). Conditions for the second round of PCR were the same as the first round, except with the JH primer (5′-GTGACCAGGGTNCCTTGGCCCCAAG-3′) replacing the CH1 primer. To confirm that there was no B-lymphocyte contamination, we studied CD19 (a B-lymphocyte marker) transcription using the same cDNA used for the amplification of the IgH V gene, and the following primer set: CD19 up, 5′-AAGGGGCCTAAGTCATTGCT-3′ (sense), and CD19 down, 5′-CACGTTCCCGTACTGGTTCT-3′ (antisense).

RT-PCR for Cell Lines and Peripheral Blood Lymphocytes—Total RNA were extracted from HT-29, HeLa S3, and peripheral blood MNC using TRIzol reagent (Invitrogen). Reverse transcription of total RNA from each of these samples was performed using a Superscript II RT kit (Invitrogen) according to the manufacturer's instructions. The human IgVH gene of the γ- or μ-chain and CD19 gene were amplified using the same PCR conditions and primers as those employed for the RT-PCR of cells obtained by LCM.

Sequencing and Analysis of Rearranged Genes—PCR products were cloned in a pGEM-T Easy Vector (Promega, Madison, WI) and sequenced with an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The V_HDJ_H sequences were compared with those found in the BLAST and Immunogenetics data bases (24) to identify the best matching germline gene segments and V(D)J junctions. V gene sequences belonging to a set of V_HDJ_H recombinants were defined on the basis of identical V_H, D_H, and J_H gene usage and V-D and D-J junction sequences. The repertoire of the cancer-derived Ig V genes was compared with that of the two healthy donors' peripheral blood lymphocytes (PBL) and published B-lymphocyte-derived Ig variable region sequences (25–28).

Determination of Taq Polymerase and Sequencing Fidelity—To analyze whether SHM was present in the cancer-derived V_H genes, we first calculated both the error rate resulting from the TaKaRa LA Taq polymerase used for PCR and from the sequencing. The results showed that in a known sequence of 17 clones generated by the same RT-PCR method and cloning procedures that were used for the V_H genes, the LA-Taq error rate was 0.07%, which equals to 0.02 mutations per V_H clone. The sequencing induced error rate was 0.

Analysis of IgVH Gene Mutations—The pattern of mutations of each sequence was compared with that of germline sequence to identify hybrid sequences derived from recombinant V_H gene segments. To analyze whether the mechanism of SHM occurring in cancer-derived Ig variable region was similar to that caused by antigen selection in B-cell-derived Ig, the mutation frequency of both the RGYW and the WRCY motifs (the mutable position is G:C, which is underlined; r = A or G, Y = C or T, and W = T or A) used as a principal hotspot for AID-induced G:U lesions was calculated (29–31). In addition, we determined the replacement-to-silent mutation (R/S) ratio in the CDRII and FWRIII regions. A V_H sequence was considered to be antigen-selected when the R/S ratio was higher than 2.9 in the CDRII and lower than 1.5 in the FWRIII region (32). The error-prone polymerases mainly induced A/T mutations that were identified as a principal site, and the dinucleotide target WA (AA or TA) mutation induced by the error-prone polymerases was involved in the mismatch repair of SHM (33–35). Therefore the WA/TW ratio was analyzed using JOINSOLVER (36).

Statistical Analysis—The distribution of V_H and J_H gene family usage and the calculation of mutations in WA *versus* TW were assessed using the Chi-squared test. Values were considered statistically significant when $p < 0.05$.

RESULTS

Cancer-derived IgVH Transcripts Were Amplified by RT-PCR—The Ig gene transcripts and repertoires were detected following LCM of cells from carcinomas from the colon, breast, oral, and lung (Fig. 1*A*) and HT-29 and HeLa S3 cell lines. Peripheral blood lymphocytes from two healthy donors served as positive controls. The rearranged V region genes of the γ and μ chains were successfully amplified and cloned from both the cancer cells and PBL (Fig. 1*B*). As expected, CD19 transcript was not detected in any of the cancer cell cDNA libraries, but was detected in PBL from the two donors (data not shown).

Functional IgVH Transcripts Were Expressed by Cancer Cells—Sequencing results from the eight cancer samples and two cancer cell lines demonstrated that 31 of the 35 $V_{H\mu}D_\mu J_{H\mu}$ sequences and 47 of the 54 $V_{H\gamma}D_\gamma J_{H\gamma}$ sequences showed functional V region gene recombination (Table 1). Among the 89 sequences examined, 11 (12.3%) were identified as nonfunctional because of mutations that introduced stop codons into the V region. This rate of nonfunctional V_HDJ_H recombination was similar to that in B-cell-derived Ig (25). The functional V region genes showed typical V_HDJ_H recombination with an N or P insert, and the J_H gene recombination occurred primarily at TG nucleotide sequences, as in B-cell-derived Ig (33). None of the 78 functional V_HDJ_H recombinations assessed was identified in the two control PBL samples or in normal B-lymphocytes, B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), or tumor-infiltrating B-lymphocytes from ductal or medullary breast carcinoma and cervical carcinoma (based on data obtained from public databases, Refs. 25–28). Some of the cancer-derived V_HDJ_H sequences identified from our study were submitted to the GenBank™ data base (GenBank™ Accession Numbers: AY270187 -AY270190, AY247234, AY286495, and AY505568).

Cancer-derived IgVH Genes Showed Restricted Pattern of V_HDJ_H Combinations—To detect usage of cancer-derived IgVH genes, we used primers that could apply for almost all V_HDJ_H rearrangements. When comparing the cancer-derived IgVH gene distribution with the best matching functional germline IgVH genes within each family, all seven of the V_H families were amplified, except V_H2. The V_H3 gene family was used most often, and the most frequently encountered germline sequences were V_H5-51, V_H6-1, V_H3-33, V_H3-15, V_H3-30, and V_H3-23, which accounted for 23.7, 18.7, 14.67, 8.0, 6.67, and 5.33% of all of the potentially possible functional cancer-derived V_H genes, respectively. The V_H5-51 and V_H6-1 frequencies were remarkably higher than expected compared with normal B-cell-derived V_H (37) and tumor-infiltrating B-cells-derived V_H (25) (Fig. 2*A*).

We measured the immunoglobulin heavy chain D_H gene distribution, and found that the D_H3, D_H6, and D_H5 sequences were most frequently used. Expression of the J_H of the IgH

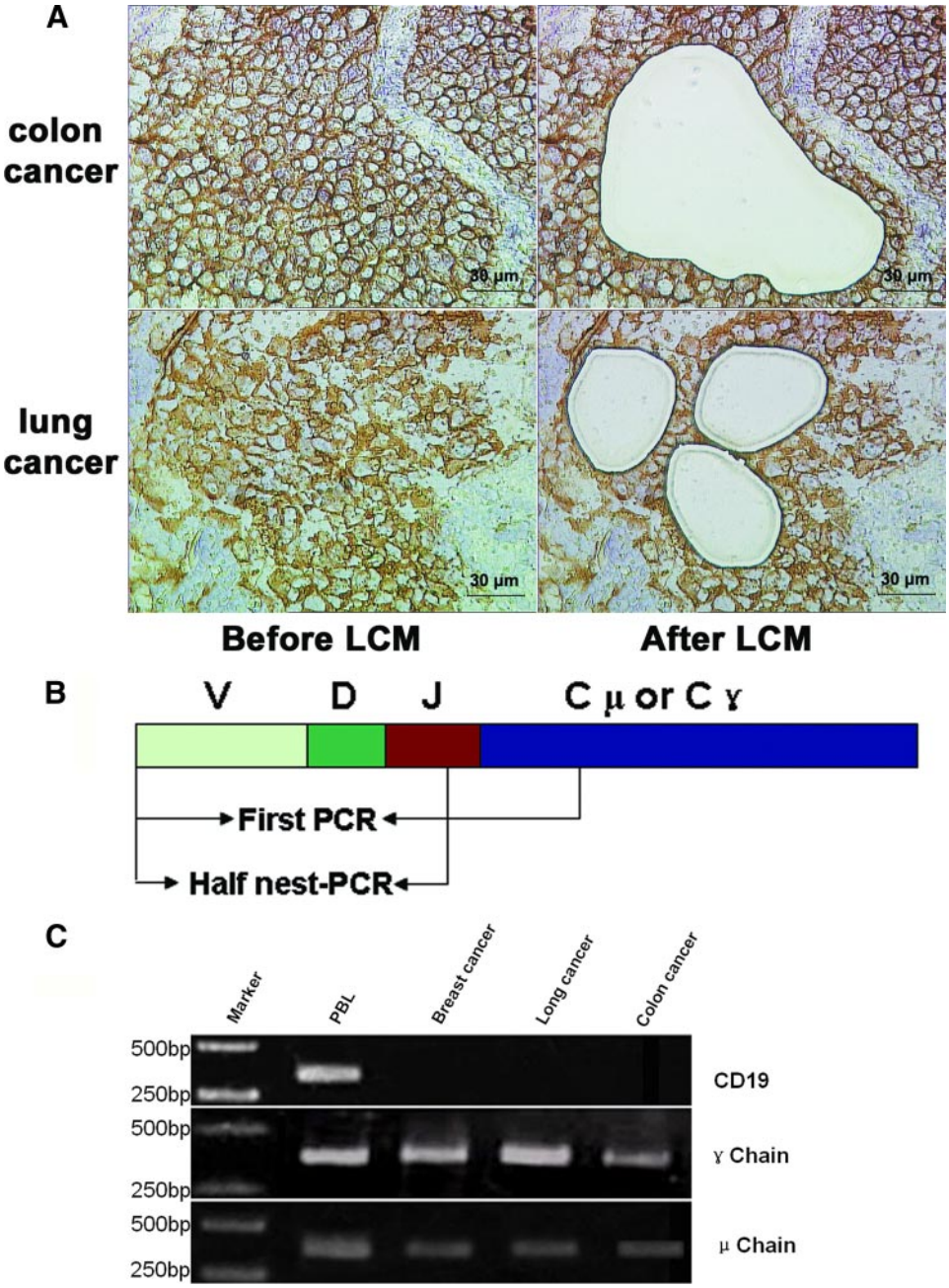

FIGURE 1. *A*, capture of the cancer cells by LCM. After frozen cancer tissue slices were stained with anti-human EpCAM antibody, about 500 $EpCAM^+$ cells per cancer nest sample were identified and dissected from squamous cell carcinoma of the lung and colon cancer tissues by LCM. *Scale bars*, 30 μm. *B*, diagrams (not to scale) of the DNA segments analyzed in this study. The *arrows* indicate the positions of the primers used for amplification of the segments. *C*, results of LCM-related RT-PCR. The variable region fragments of Ig γ chain and μ chain were amplified. CD19 was used as positive control for B-lymphocytes.

showed that J_H4 was expressed most often (72.7%), although all six J_H genes were detected (Fig. 2*B*).

Several Sets of Predominant Functional V_HDJ_H Gene Recombinations Were Detected in Human Cancer Cells—A noteworthy finding in this study was that the cancer-derived Ig gene repertoire exhibited distinct characteristics. LCM-isolated cancer cells usually expressed one to three sets of dominant V_HDJ_H recombination patterns, as well as some individual V_HDJ_H recombination (Table 2). Notably, seven sets of predominant V_HDJ_H recombinations were detected in more than one cancer cell type (Table 3). Of the 26 $V_{H\mu}D_\mu J_{H\mu}$ sequences studied, three sets of V_HDJ_H recombinations were predominant: V_H3-15/D3-10/J_H4, found in 5 of 26 (19.2%) cases of carcinomas from the breast and colon; V_H6-1/D6-13/J_H4, found in 5 of 26 (19.2%) cases of carcinomas from the lung and oral cavity; and V_H4-30-2/D3-22/J_H4, found in 4 of 26 (15.4%) cases of carcinomas from the breast and colon. Of the 41 $V_{H\gamma}D_\gamma J_{H\gamma}$sequences studied, the following types of V_HDJ_H recombinations were predominant: V_H5-51/D3-9/J_H4 in 19 of 38 (50%) cases of carcinomas of the breast, colon, oral, and lung; and V_H3-30/D6-19/J_H4 in 4 of 38 (10.5%) cases of carcinomas of the breast, colon, and lung. There was no correlation between the patterns of V_HDJ_H recombinations and the histological origins of the cancers.

In contrast, all of the $V_{H\gamma}D_\gamma J_{H\gamma}$ sequences obtained, either from the HT-29 cell line (7 clones) or from the HeLa S3 cell line (6 clones), showed distinct diversity. None of the γ chain clones from the cell lines was detected in any of the LCM-isolated cancer cells. All six $V_{H\mu}D_\mu J_{H\mu}$ sequences from HT-29 cells showed identical V_H6-1/D6-13/J_H4 recombination, which was also detected in LCM-isolated carcinomas of the lung and oral cavity. All three $V_{H\mu}D_\mu J_{H\mu}$sequences from HeLa S3 cells showed identical V_H3-30-3/D6-19/J_H5 recombination, which was not identified in the LCM-isolated cancer cells. Ten V_HDJ_H recombination sequences in two control PBL samples exhibited distinct diversity (Table 2).

Mechanism of SHM of Functional V Genes in Cancer Cells Was Different from Antigen Selection in B-cell-derived V_HDJ_H Sequences—Somatic hypermutation of the VH region is known to be an important event in B-cell-derived Ig following antigen stimulation. Mutational analysis demonstrated that all of the Vμ gene sequences showed fewer than 5% mutations, and 73.9% of Vμ gene sequences had fewer than 2% mutations, which should be considered as "unmutated" by definition (38). In contrast, most functional $V_{H\gamma}$ sequences were highly mutated. Approximately 90.6% of $V_{H\gamma}$ sequences had greater than 5% mutation, which should be considered as "mutated" by definition (38) (Table 2). Unexpectedly, within a set of V_HDJ_H recombinant, the V regions from different cancer cell types showed identical

V_HDJ_H recombination and junction. The recombination was either identical or differed only by several mutation targets over the V_H region. For example, within the V_H5-51/D3-9/J_H4 recombination set, 19 of the V_H5-51 sequences showed almost identical mutation targets, which were identical to our previously published V_H5-51/D3-9/J_H4 recombination sequence described for lung caner cells (Fig. 3) (20). Despite the high mutation rate, the rate of homology within the set of V_HDJ_H recombinants ranged from 92.7 to 100%.

TABLE 1

Rate of functional rearrangement in 89 V_HDJ_H recombinations from different cancer types

Sample origin	Ig type	No. of sample	No. of clone	No. of functional V_HDJ_H recombination	No. of non-functional V_HDJ_H recombination
PBL	γ chain	2	6	5	1
	μ chain	2	4	4	0
	Total	4	10	9	1 (10%)[a]
Breast cancer	γ chain	3	12	10	2
	μ chain	3	10	10	0
Lung cancer	γ chain	1	6	6	0
	μ chain	1	2	2	0
Colon cancer	γ chain	2	11	10	1
	μ chain	1	5	4	1
Oral carcinoma	γ chain	2	12	10	2
	μ chain	1	9	7	2
HT29 cell	γ chain	1	7	7	0
	μ chain	1	6	5	1
Hela S3 cell	γ chain	1	6	4	2
	μ chain	1	3	3	0
Total (cancer)		18	89	78	11 (12%)

[a] The percentage of all sequences corresponding to each group of genes is shown in parentheses.

In B-cell-derived Ig, the mutations may either be silent or missense that changes the affinity of the Ig for the antigen; the latter may occasionally give rise to cells expressing higher affinity antibodies, usually with mutations clustered in the CDRs and as a result of antigen selection. Moreover, mutations introduced by AID activity under antigen selection typically target the known SHM hotspot, WRCY, and its complement, RGYW. Furthermore, the mutation frequency is expected to be higher in the CDRs than in the framework regions (FWRs). However, no AID transcript was detected by RT-PCR in the LCM-isolated cancer cells. In contrast, we detected an AID transcript in HT-29 cell line at a low level (Genbank™ Accession Number, AY748364), as well as in Raji cell line (B-lymphocyte-derived, Burkitt's lymphoma) which was used as a positive control (data not shown). In addition, in only 17 of 43 (39.5%) $V_{H\gamma}$ sequences were there more frequent mutations in the RGYW hot spot. In 3 of 43 $V_{H\gamma}$ sequences, the mutation frequency was higher in the CDRs than in the FWRs. In only 7 of 43 (16.3%) $V_{H\gamma}$ sequences were the R/S ratios in the CDRII and FWRIII >2.9 and <1.5, respectively. In the V_H5-51/D3-9/J_H4 recombination set, a higher mutation frequency, ~40%, occurred in the RGYW motif (if the ratio was under 25.6%, it was not considered classical SHM) (Fig. 3). In contrast, the mutations in cancer-derived Ig mainly occurred in the FWRIII and not in the CDRs. This finding suggests that a large number of the mutations introduced into the cancer-derived $V_{H\gamma}$ sequences were not typical of B-cell-derived V_H mutations caused by antigen selection.

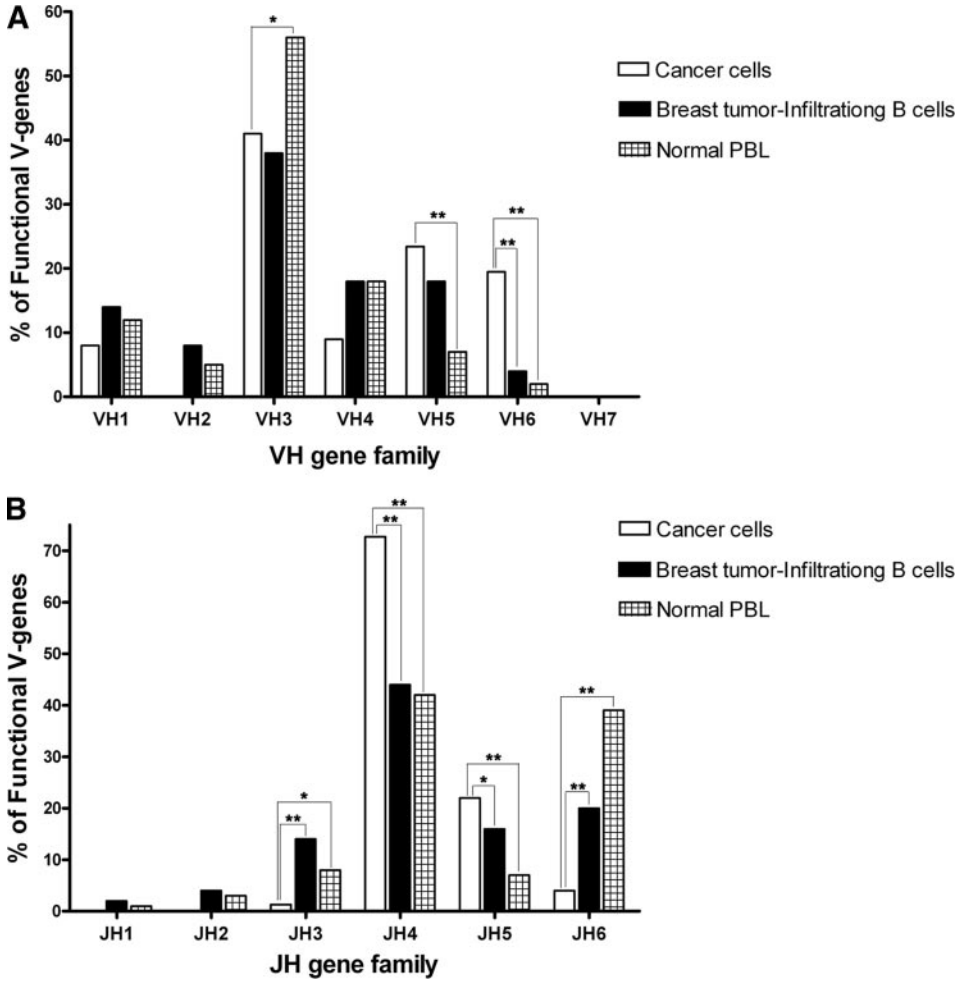

FIGURE 2. **V_H (*A*) and J_H (*B*) gene family usage profiles of cancer-derived Ig compared with the normal PBL-derived Ig.** We analyzed the usage of 78 functional V_H genes from the cancer-derived Ig and collected five groups of usage profiles from healthy donors PBL-derived Ig from previous studies (22, 23, 25, 26, 33). The V_H and J_H gene family usage data from the combined patient group differed significantly from that of the healthy donors PBL-derived Ig ($p < 0.05$ for both V_H and J_H).

To define the role of the error-prone polymerase in cancer-derived $V_{H\gamma}$ hypermutation, we analyzed the mutation frequency in the WA and TW motifs, and found that mutation of the WA motif (36/43 $V_{H\gamma}$ sequences) was significantly more frequent than that of the TW motif (11/43 $V_{H\gamma}$ sequences, $p <$ 0.001). These data reveal a strand bias and suggest that error-prone polymerases are involved in the mutation of cancer-derived $V_{H\gamma}$ sequences. The mutational frequency in the WRCY/RGYW

TABLE 2

Assignment of the likely matching germline variable region genes to the V_HDJ_H recombinants from different cancer types and analysis of the V gene somatic mutation rate

Cases	No. of clones	$V_{H\gamma}DJ_{H\gamma}$ (no. of clone)	Mutation rate	No. of clones	$V_{H\mu}DJ_{H\mu}$ (no. of clone)	Mutation rate
Breast cancer (case 1)	5	V_H5-51/D3-9/JH4(3)	15.6% (3)	3	VH4-30-2/D3-22/JH4(1)	3.3% (1)
		VH7-4-1/D2-8JH4(1)	1% (1)		VH3-33/D3-10/JH4(1)	2.2% (1)
		VH3-9/D3-9/JH3(1)	stop[a] (1)		VH3-33/D5-12/JH5(1)	0 (1)
Breast cancer (case 2)	5	VH5-51/D3-9/JH4(4)	15.6% (2); 17.5% (1)	3	VH4-30-2/D3-22/JH4(1)	0 (1)
			stop (1)		VH3-33/D5-12/JH5(1)	4.4% (1)
		VH1-16/NI/JH4(1)	stop (1)		VH3-15/D3-10/JH4(1)	1.1% (1)
Breast cancer (case 3)	2	VH5-51/D3-9/JH4(1)	15.6% (1)	4	VH4-30-2/D3-22/JH4(1)	3.3% (1)
					VH3-33/D5-12/JH5(1)	0 (1)
		VH3-30/D6-19/JH4(1)	13.5% (1)		VH4-15/D6-13/JH4(1)	10% (1)
					VH4-61/D6-13/JH4(1)	0 (1)
Lung cancer	6	VH5-51/D3-9/JH4(2)	15.6% (2)	2	VH6-1/D6-13/JH4(1)	3.3% (1)
		VH3-30/D6-9/JH4(2)	12.5% (2)		VH6-1/D1-7/JH6(1)	2.1% (1)
		VH1-3/D5-12/JH4(2)	28.1% (2)			
Colon cancer (case 1)	4	VH5-51/D3-9/JH4(4)	15.6% (4)	5	VH3-15/D3-10/JH4(4)	3.5% (4)
					VH4-30-2/D3-22/JH4(1)	stop (1)
Colon cancer (case 2)	7	VH5-51/D3-9/JH4(4)	15.6% (3), 17.5% (1)	nd[b]	nd	nd
		VH3-30/D6-9/JH4(1)	13.5% (1)			
		VH1-69/D6-15/JH4(1)	stop (1)			
		VH7-4-1/D3-10/JH4(1)	4% (1)			
Oral cancer (case 1)	3	VH5-51/D3-9/JH4(2)	15.6% (1), stop (1) stop (1)	9	VH6-1/D6-13/JH4(4)	0 (4)
		VH1-2/D2-8/JH4(1)			VH6-1/D1-7/JH6(3)	7.6% (3)
					VH6-1/D2-2/JH4(2)	2.5% (2)
Oral cancer (case 2)	9	VH3-33/D6-19/JH5(6)	11.5% (5);	nd	nd	nd
		VH3-23/D1-26/JH4(3)	16.3% (1) 7.3% (3)			
HT-29	7	VH5-51/D2-2/JH4(1)	15.% (1)	6	VH6-1/D6-13/JH4(6)	3.3% (4), 1% (1), 7.6% (1)
		VH3-33/D3-10/JH5(1)	5% (1)			
		VH1-8/D5-18/JH5(1)	18% (1)			
		VH1-69/D1-26/JH5(1)	stop (1)			
		VH3-23/D2-15/JH4(1)	7.1% (1)			
		VH3-30/D6-13/JH4(1)	9.2% (1)			
		V1-3/D3-10/JH5(5)	8.3% (1)			
HelaS3	6	VH1-8/D1-14/JH4(1)	8.1% (1)	3	VH3-30-3/D6-19/JH5(3)	1.5% (3)
		VH1-8/D2-2/JH4(1)	6.2% (1)			
		VH4-59/D3-10/JH4(1)	10.4% (1)			
		VH4-59/D3-22/JH4(1)	7.5% (1)			
		VH3-30/N1/JH5(1)	stop (1)			
		VH3-7/D3-10/JH4(1)	9.1% (1)			
PBL	3	VH3-23/D3-10/JH4(1)	4.8% (1)	4	VH3-21/D1-26/JH4(1)	0 (1)
					VH4-59/D1-20/JH4(1)	4.8% (1)
(case 1)		VH1-69/D5-5/JH4(1)	11.5% (1)		VH4-39/D3-10/JH5(1)	2.9% (1)
		VH1-8/D2-2/JH5(1)	12.5% (1)		VH3-23/D6-19/JH5(1)	14.4% (1)
PBL	3	VH4-59/D3-3/JH4(1)	20% (1)	nd	nd	nd
		VH4-59/D4-17/JH4(1)	17.9% (1)			
(case 2)		VH3-43/NI/JH5 (1)	3.1% (1)			

[a] Stop, non-functional V_HDJ_H sequences.
[b] nd, not done.

region, the WA/TW motifs, and calculations of the R/S mutation ratio in CDRII and FWRIII are summarized in Table 4.

Cancer-derived $V_{H\gamma}D_\gamma J_{H\gamma}$ Sequences Did Not Appear to Originate from the Classical Class Switching—In general, the B-cell-derived V_HDJ_H recombination pattern of the γ chain is similar to that of the μ chain (the precursor of γ chain), because class switching changed only the constant region sequence from the μ chain to the γ chain. However, there was no identical pattern between $V_{H\gamma}D_\gamma J_{H\gamma}$ and $V_{H\mu}D_\mu J_{H\mu}$ in any of the cases studied, although each sample expressed a restricted $V_{H\gamma}D_\gamma J_{H\gamma}$ or $V_{H\mu}D_\mu J_{H\mu}$ recombination pattern (Table 2). This suggests that cancer-derived $V_{H\gamma}D_\gamma J_{H\gamma}$ sequences do not originate from the classical class switching.

DISCUSSION

B-lymphocytes have been considered the primary source of serum Ig. However, we have found that cells from epithelial cancer and hyperplasia could also express Ig (17–18, 21). In this study, we confirmed that functional Ig gene recombination and transcription occurred in a variety of cancer types. Our analysis of 78 functional cancer-derived V_HDJ_H sequences showed that cancer-derived Ig shared some features with B-cell-derived Ig, such as V_HDJ_H recombination, insertion of the N region into junctions, and J_H gene recombination at TG nucleotide sequences. In addition, there were either low levels or a complete absence of mutations in the $V_{H\mu}D_\mu J_{H\mu}$ sequences and high levels of mutations in the functional $V_{H\gamma}D_\gamma J_{H\gamma}$ sequences.

On the other hand, cancer-derived V_HDJ_H recombinations also exhibited distinct features that differed from B-cell-derived Ig recombination. For example, cancer-derived Ig V_H, D, and J_H usage showed distinct preferences, such as that V_H5-51 and V_H6-1 frequencies were higher and that J_H4 was expressed most often. The primers used in this experiment were designed to be applicable to almost all V_HDJ_H recombinations; however, we found that V_H5-51 and V_H6-1 recombination frequency was significantly higher than expected compared with normal B-cell-derived V_H (22, 26–28).

It is known that each B-cell expresses a unique V_HDJ_H recombination, including a random N region sequence. Therefore, the likelihood of identical junction sequences from two independent B-cell clones occurring in an individual should be lower than 1 in 4 million. Unexpectedly, several restricted

TABLE 3

Seven sets of predominant V_HDJ_H recombinants found in different cancer types

The clone name appears in four letters as follows: the first represents the tissue of origin (B for breast cancer, L for lung cancer, C for colon cancer, O for oral cancer, and H for HT-29 cell line); the second represents immunoglobulin isotype (G for IgG, and M for IgM); the third represents the patient number; and the last number in brackets represents the number of clones in this set of V_HDJ_H rearrangements.

No. of sets	Clone	V gene name	V region	N1	P	D region	N1	P	J region	J gene name	D gene name
1	BG1 (3)	IGHV5-51*01	tgtgcgaga	tgggatg		.....ttatgattggatttata..	cagc		.....tgactactgg	IGHJ4*02	IGHD3-9*01
	BG2 (4)	IGHV5-51*01	tgtgcgaga	tgggatg		.....ttatgattggatttata..	cagc		.....tgactactgg	IGHJ4*02	IGHD3-9*01
	BG3 (1)	IGHV5-51*01	tgtgcgaga	tgggatg		.....ttatgattggatttata..	cagc		.....tgactactgg	IGHJ4*02	IGHD3-9*01
	CG1 (4)	IGHV5-51*01	tgtgcgaga	tgggatg		.....ttatgattggatttata..	cagc		.....tgactactgg	IGHJ4*02	IGHD3-9*01
	CG2 (4)	IGHV5-51*01	tgtgcgaga	tgggatg		.....ttatgattggatttata..	cagc		.....tgactactgg	IGHJ4*02	IGHD3-9*01
	LG1 (1)	IGHV5-51*01	tgtgcgaga	tggaatg		.....ttatgattggatttata..	cagc		.....tgactactgg	IGHJ4*02	IGHD3-9*01
	OG1 (2)	IGHV5-51*01	tgtgcgaga.	tgggatg		.....ttatgattggatttata..	cagc		.....tgactactgg	IGHJ4*02	IGHD3-9*01
2	BG3 (1)	IGHV3-30*01	tgtgccagaga			.....aacaatggctg....		cctc	....ctttgacaattgg	IGHJ4*03	IGHD6-19*01
	LG1 (2)	IGHV3-30*01	tgtgccagaga			.....aacaatggctg....		cctc	....ctttgacaattgg	IGHJ4*03	IGHD6-19*01
	CG2 (1)	IGHV3-30*01	tgtgccagaga			.....aacaatggctg....		cctc	....ctttgacaattgg	IGHJ4*02	IGHD6-19*01
3	HM (3)	IGHV6-1*01	tgtgcaagaga		at	.....tagcagcagct.....			.....tttgactactgg	IGHJ4*03	IGHD6-13*01
	OM1 (2)	IGHV6-1*01	tgtgcaagaga		at	.....tagcagcagct.....			.....tttgactactgg	IGHJ4*02	IGHD6-13*01
	LM (1)	IGHV6-1*01	tgtgcaagaga		at	.....tagcagcagct.....			.....tttgactactgg	IGHJ4*02	IGHD6-13*01
	OM2 (2)	IGHV6-1*01	tgtgcaagaga		at	.....tagcagcagct.....			.....tttgactactgg	IGHJ4*02	IGHD6-13*01
4	BM1 (1)	IGHV4-30-2*01	tgtgccgg...	cc		.....gaagtggttattact	c		.ccccttgactactgg	IGHJ4*02	IGHD3-22*01
	BM2 (1)	IGHV4-30-2*01	tgtgccgg...	cc		.....gaagtggttattact	c		.ccccttgactactgg	IGHJ4*02	IGHD3-22*01
	BM3 (1)	IGHV4-30-2*01	tgtgccgg...	cc		.....gaagtggttattact	c		.ccccttgactactgg	IGHJ4*02	IGHD3-22*01
5	BM21 (1)	IGHV3-15*01	tgtaccacaaa	cctga	ac	gtattactatggttcggggaccga...		acccccc	.....gactactgg	IGHJ4*02	IGHD3-10*01
	CM1 (4)	IGHV3-15*01	tgtaccacaaa	cctga	ac	gtattactatggttcggggaccga...		acccccc	.....gactactgg	IGHJ4*02	IGHD3-10*01
6	LM1 (1)	IGHV6-1*02	tgtgcaagag.			ggcgtacgtgg.....c		ccagc	.....ggacgtctgg	IGHJ6*02	IGHD1-7*01
	OM1 (3)	IGHV6-1*01	tgtgcaagag.			ggcgtacgtgg.....c		ccagc	.....ggacgtctgg	IGHJ6*02	IGHD1-7*01
7	BM2 (1)	IGHV3-33*01	tgtgcgagaga			.....tggggtggctacgattac	aaggaggtg		....ctggttcgacccctgg	IGHJ5*02	IGHD5-12*01
	BM3 (1)	IGHV3-33*01	tgtgcgagaga			.....tgggggtggctacgattac	aaggaggtg		....ctggttcgacccctgg	IGHJ5*02	IGHD5-12*01

V_HDJ_H sequences were identified in each cancer sample. More interestingly, different cancer samples showed identical V_HDJ_H recombination patterns, with identical junctions and V_H region mutation targets. Several dominant V_HDJ_H recombination sets were frequently expressed in different cancer types. These results suggest that there is an unknown mechanism allowing epithelial cancer cells to express several repeated sets of dominant V_HDJ_H sequences. We eliminated the possibility of cross-contamination among cancer samples in a number of ways. First, using RT-PCR, the control tube (containing no cDNA) did not show a positive band when the V_HDJ_H sequences were amplified. Second, there were no samples with identical Ig heavy chain gene repertoires among the eight cancer samples, suggesting that there was no cross contamination among different samples. In contrast, both HT-29 and HeLa S3 cells showed monoclonal characteristics in the $V_{H\mu}D_\mu J_{H\mu}$. The $V_{H\gamma}D_\gamma J_{H\gamma}$ derived from these two cancer cell lines showed distinct diversity, which was similar to that of B-cells, but different from primary cancer cells. Identical V_HDJ_H sequences were not detected between the two cancer cell lines, which implied that the genetic characteristics of the cancer cell lines had changed under long-term culture *in vitro*.

The mechanism of SHM is another distinct feature in cancer-derived $V_{H\gamma}D_\gamma J_{H\gamma}$ sequences as opposed to B-cell-derived $V_{H\gamma}D_\gamma J_{H\gamma}$ sequences. In B-cell-derived Ig V regions, mutations induced by antigen selection occurred more frequently in the CDRs than in the FWRs. Moreover, there is a higher R/S mutation ratio in the CDRs. However, only a few cancer-derived $V_{H\gamma}D_\gamma J_{H\gamma}$ sequences matched the pattern of antigen selection. In addition, AID is thought to be necessary for SHM of the Ig gene, since it can deaminate C to U on both DNA strands, resulting in the symmetrical mutation of C on both strands. The AID enzyme site preference results in hypermutation of the RGYW hotspot motif. In this study, only the V_H5-51/D3-9/J_H4 pattern matched the AID-induced R*G*YW hypermutation pattern. Moreover, mutations in V_H5-51 frequently occurred in the FWRIII, but not in the CDRs. Additionally, no AID transcript was detected by RT-PCR in the LCM-isolated cancer cells. In contrast, we detected AID transcripts in the HT-29 cell line at a low level, as well as in Raji cell line. It is possible that AID expression is unnecessary for Vγ SHM in cancer cells and that other mechanisms may be involved in SHM of the cancer-derived Ig V region. Babbage *et al.* recently demonstrated constitutive expression of AID in six breast cancer cell lines and Matsumoto *et al.* demonstrated expression of AID in stomach cancer cells (22, 39). The mutational bias of A *versus* T in Ig genes results from DNA pol η activity (40), which functions as a secondary mutator (41, 42). Thus, the excess of mutations in the WA motif suggests that DNA pol η may be involved in mutating A:T base pairs in the Ig V gene in cancer cells.

The third distinct feature that differentiates cancer-derived from B-cell-derived $V_{H\gamma}D_\gamma J_{H\gamma}$ sequences is that the cancer-derived $V_{H\gamma}D_\gamma J_{H\gamma}$ sequences do not seem to originate from classical class switching. Class switch recombination and SHM are two important events in Ig production. In the classical Ig class switching, the IgM-producing cells are precursors of the IgG producers, and the Vγ gene assembly mode should be similar to that of $V_{H\mu}D_\mu J_{H\mu}$. Although

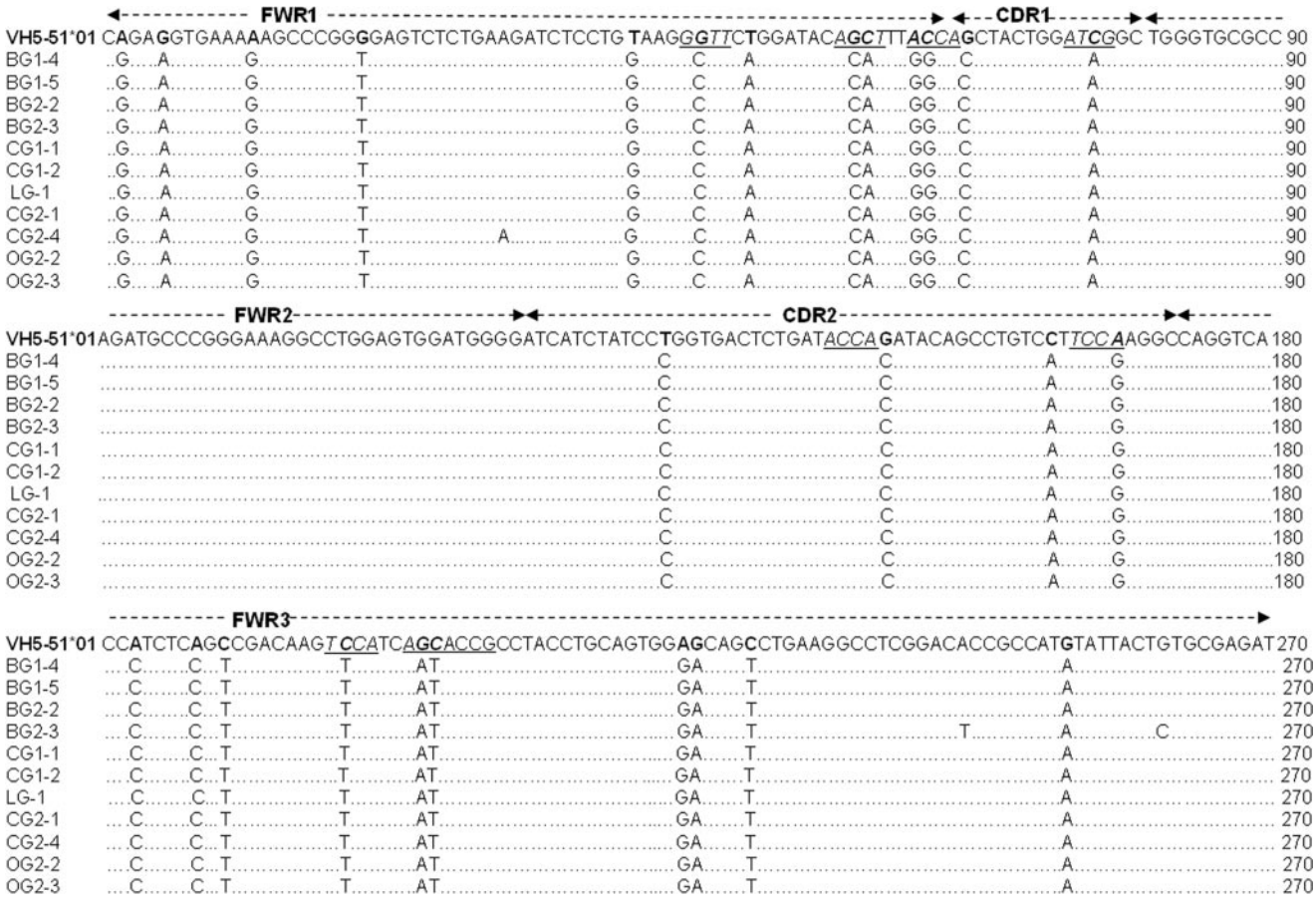

FIGURE 3. **The analysis of V_H5-51/D3-9/J_H4 recombinations derived from different cancer types.** These $V_{H\gamma}D_\gamma J_{H\gamma}$ sequences showed almost identical mutation targets over the V segment. Somatic mutations were identified by comparison with the published germline V_H5-51 gene in NCBI. The RGYW/WRCY motif was *underlined*. The first 25 nucleotides were excluded from the analysis because they were encoded by the primers. The clone name appears in four characters: the first character represents the tissue origin (*B* for breast cancer, *L* for lung cancer, *C* for colon cancer, and *O* for oral cancer); the second represents the immunoglobulin isotype (*G* for IgG); the third represents the patient number, and the last represents the clone number.

$V_{H\mu}D_\mu J_{H\mu}$and $V_{H\gamma}D_\gamma J_{H\gamma}$ could be synchronously detected in the same cancer cells in this study, we did not detect any identical patterns of $V_{H\gamma}D_\gamma J_{H\gamma}$ and $V_{H\mu}D_\mu J_{H\mu}$ in the cases studied. This unexpected result suggested that the IgGs were completely different from the IgMs in the same cancer cells, and that IgG production in these cells did not follow the classical class switching mechanism. These results raised the possibility that IgGs might be coded by an allele on another chromosome. Our finding (by Southern blot analysis using a J_H DNA segment probe) that two Ig alleles in HT-29 had been rearranged (data not shown) supported the presence of such a mechanism. Alternatively, there may be another class switching mechanism. Jhagvaral *et al.* (36) recently described that naive B-lymphocytes could develop into IgG-secreting cells through successive cell divisions.

In this study, a pivotal precaution was to avoid B-cell contamination. B-lymphocytes and plasma cells are capable of infiltrating cancer tissues and are mainly located in the stroma. By using the LCM method, we specifically captured the $EpCAM^+$ cancer cells in the cancer nest regions and avoided capturing tumor-infiltrating B-lymphocytes and plasma cells. We did not detect B-cell contamination in any of the cDNA libraries from the LCM-isolated cancer cells. Importantly, none of the cancer-derived V_HDJ_H sequences were homologous to the V_HDJ_H recombination sequences of the two control PBL samples or published recombination sequences present in B-lymphocytes and B-cell CLL/SLL. Additionally, there was no sequence homology with published sequences for tumor-infiltrating B-lymphocytes in breast cancer tissues (25). This lack of recombinant sequence homology with B-lymphocytes indicates that the detected Ig sequences are specific to epithelial cancer cells.

To be reactive to multiple antigens, B-cells generate Ig diversity through several mechanisms. However, the biological significance of non-B-cell-derived Ig is not yet clear. The cancer cell-derived Ig repertoire displayed distinct features suggesting that non-B-cell-derived Ig may have important undiscovered activities because of their diverse origins. We previously noted that Ig expression and activity in cancer cell lines could be blocked by specific antisense DNAs and antibodies, causing the cancer cells to undergo apoptosis (20). These data suggest that non-B-cell-derived Ig is involved in the growth and survival of cells.

In summary, many nonhematopoietic tumor cells express Ig. The cancer-derived Ig gene repertoire displays several distinct characteristics, suggesting that there is an idiosyncratic mechanism for cancer-derived Ig gene expression. The gene expression patterns of Ig in different cancer cells may prove useful in

TABLE 4

Summary of the mutation frequency in WRCY/RGYW and WA/TW motifs and calculation of the R/S mutation ratios occurring in CDRII and in FWRIII

Clone	Mutation frequency in RGYW/WRCY	Mutation in WA/TW		R/S ratio in CDRII and FWRIII	
		WA	TW	CDRII	FWRIII
	%				
BG1-1	62.5 (≥25)	3	0	0/1	9/4
BG1-2	16.7	3	0	2/0 (>2.9)	3/0
BG1-3	0.0	0	1	0/0	0/1 (<1.5)
BG1-4	62.5 (≥25)	2	0	0/1	9/4
BG1-5	62.5 (≥25)	2	0	0/1	9/4
BG2-1	62.5 (≥25)	3	1	0/1	13/6
BG2-2	62.5 (≥25)	2	0	0/1	9/4
BG2-4	62.5 (≥25)	2	0	0/1	11/4
BG2-5	62.5 (≥25)	2	0	0/1	9/4
BG3-2	18.2	3	2	4/0 (>2.9)	4/7 (<1.5)
CG1-1	62.5 (≥25)	2	0	0/1	9/4
CG1-2	62.5 (≥25)	2	0	0/1	8/4
CG1-3	62.5 (≥25)	2	0	0/1	9/4
CG1-4	62.5 (≥25)	2	0	0/1	9/4
CG2-2	0.0	0	0	0/0	0/0
CG2-3	18.2	3	2	4/0 (>2.9)	4/7 (<1.5)
CG2-4	62.5 (≥25)	2	1	0/1	9/4
LG1-1	18.2	3	2	4/0 (>2.9)	4/7 (<1.5)
LG1-2	18.2	3	2	4/0 (>2.9)	4/7 (<1.5)
LG1-3	14.3	4	0	2/1	8/5
LG1-4	14.3	5	1	2/1	8/5
LG1-5	62.5 (≥25)	22	0	0/1	9/4
LG1-6	62.5 (≥25)	0	2	0/1	9/4
OG1-2	62.5 (≥25)	0	2	0/1	9/4
OG2-1	9.1 (≥25)	1	2	5/2	5/3
OG2-2	23.1	0	1	3/0 (>2.9)	1/3 (<1.5)
OG2-3	23.1	0	1	3/0 (>2.9)	1/3 (<1.5)
OG2-4	16.7	1	2	6/1 (>2.9)	5/2
OG2-5	16.7	1	2	6/1 (>2.9)	5/2
OG2-6	16.7%	1	2	6/1 (>2.9)	5/2
OG2-7	16.7	1	2	6/1 (>2.9)	5/2
OG2-8	16.7	1	2	6/1 (>2.9)	5/2
OG2-9	16.7	1	2	6/1 (>2.9)	5/2
SG1-1	18.2	5	3	3/1 (>2.9)	13/5
SG1-2	45.5 (≥25)	6	3	3/0 (>2.9)	10/6
SG1-3	10.0	0	0	0/0	0/2 (<1.5)
SG1-6	0.0	2	1	1/0 (>2.9)	7/4
HG1-1	12.5	0	1	1/1	8/0
HG1-2	7.7	0	0	0/0	0/0
HG1-3	25.0 (≥25)	3	4	3/3	10/3
HG1-4	33.3 (≥25)	5	3	7/3	7/8 (<1.5)
HG1-5	15.4	2	1	2/1	4/0
HG1-7	21.4	2	2	3/0 (>2.9)	7/3
		95	35		
	20/43 of Vγ rearrangements showed ≥25% mutation frequency in RGYW/WRCY motifs	36/43 of Vγ rearrangements showed excess mutations in WA		8/43 of Vγ rearrangements showed both R/S >2.9 in CDRII and R/S <1.5 in FWRIII	

understanding the structure and function of nonhematopoietic-derived Ig. In addition, these findings may have important implications for the diagnosis, targeted therapy, as well as monitoring of residual disease of cancers.

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