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# Genomic Organization and Expression Analysis of B7-H4, an Immune Inhibitory Molecule of the B7 Family<sup>1</sup>

In-Hak Choi,<sup>2\*§</sup> Gefeng Zhu,<sup>2\*§</sup> Gabriel L. Sica,<sup>\*</sup> Scott E. Strome,<sup>†</sup> John C. Cheville,<sup>‡</sup> Julie S. Lau,<sup>\*</sup> Yuwen Zhu,<sup>\*</sup> Dallas B. Flies,<sup>\*</sup> Koji Tamada,<sup>\*</sup> and Lieping Chen<sup>3\*</sup>

**B7-H4 is a recently identified B7 family member that negatively regulates T cell immunity by the inhibition of T cell proliferation, cytokine production, and cell cycle progression. In this study, we report that the genomic DNA of human B7-H4 is mapped on chromosome 1 comprised of six exons and five introns spanning 66 kb, of which exon 6 is used for alternative splicing to generate two different transcripts. Similar B7-H4 structure is also found in mouse genomic DNA in chromosome 3. A human B7-H4 pseudogene is identified in chromosome 20p11.1 with a single exon and two stop codons in the coding region. Immunohistochemistry analysis using B7-H4-specific mAb demonstrates that B7-H4 is not expressed on the majority of normal human tissues. In contrast, up to 85% (22 of 26) of ovarian cancer and 31% (5 of 16) of lung cancer tissues constitutively express B7-H4. Our results indicate a tight regulation of B7-H4 expression in the translational level in normal peripheral tissues and a potential role of B7-H4 in the evasion of tumor immunity. *The Journal of Immunology*, 2003, 171: 4650–4654.**

Outcome of TCR signal upon ligation by its cognate Ag is tightly controlled by both positive and negative regulatory events. There is accumulated evidence that costimulatory molecules, upon engaging their cognate counterreceptors, play a critical role in fine-tuning T cell responses. Ligation of CTLA-4 on activated T cells has a profound effect on the inhibition of their growth and functions (1). Similarly, engagement of programmed death-1 on T and B cells is believed to induce a negative signal for the inhibition of T cell responses (2, 3). It appears that peripheral tissues are equipped with a group of such negative costimulatory ligands. B7-H1 (4), one of the ligands for programmed death-1, is inducible in many peripheral organs and tissues and constitutively expressed on the majority of human cancers (5) and cancer-associated myeloid dendritic cells (6). Ligation of activated T cells by B7-H1 undergoes increased programmed cell death (5) or inhibition (7). Therefore, the expression of negative costimulatory molecules on peripheral tissues may be critical for the control of immune responses in the effector phase.

We have recently identified and characterized a new B7 family member, B7-H4, with potent inhibitory functions in T cell responses (8). B7-H4 mRNA expression was found to be widely distributed in the peripheral tissues including kidney, liver, lung, spleen, thymus, and placenta. However, B7-H4 protein expression on the cells seems to be limited and has an inducible mode in hemopoietic cells. Sensitive RT-PCR analysis indicates that human tissues contain at least two different transcripts. Immobilized

or cell surface B7-H4 could inhibit the proliferation of T cells experiencing Ag stimulation. In addition, B7-H4 was known to suppress the production of both Th1- and Th2-derived cytokines such as IFN- $\gamma$ , IL-2, IL-4, and IL-10 from the naive T cell. Soluble B7-H4 protein also impedes the proliferation of T cells. In contrast, blockade of endogenous B7-H4 by neutralizing mAb enhances Ag-specific T cell responses against allogeneic Ags in vivo. Preliminary data indicate that engagement by B7-H4 on receptors on activated T cells has a minimal effect on programmed cell death but has a profound effect on cell cycle arrest, by which most of the T cells activated by anti-CD3 are arrested in the G<sub>0</sub>/G<sub>1</sub> phase. Therefore, B7-H4 may play a role in negative regulation of T cell immunity in peripheral tissues.

In this report, we describe the analysis of the *B7-H4* gene, including its chromosomal assignment and genomic structure, as well as the structural analysis of transcripts and the expression of B7-H4 protein in cancer tissues and cell lines.

## Materials and Methods

### *Analysis of genomic structure of B7-H4*

The human genomic database from the National Center for Biotechnology Information (NCBI) was queried with B7-H4 cDNA for the chromosomal assignment. Genomic structure was analyzed by comparing the B7-H4 cDNA sequence to the working draft sequence retrieved from human genome using the BLAST program of NCBI. The amino acid sequences and phylogeny tree of the B7 family were analyzed using the ClustalW algorithm with the BLOSUM 30 matrix (MacVector; Oxford Molecular Group, Beaverton, OR).

### *PCR analysis*

For the tissue distribution of B7-H4 message, multiple tissue cDNA panels (Clontech Laboratories, Palo Alto, CA) were used for PCR analysis of B7-H4 expression as described previously (8, 9). Amplification of B7-H4 was performed using forward primer (5'-TCCATCACAGTCACTACTGTCGCCTCAG-3') and reverse primer (5'-GCTCCCCTCTTCCAGGCCTTTTCTACTC-3') derived from the IgV and 3' untranslated region (UTR)<sup>4</sup> of human (h)B7-H4, respectively. Following amplification, PCR products were analyzed by agarose (2%) gel electrophoresis, purified with a gel extraction kit (Qiagen, Valencia, CA), and sequenced using the same primers as used for PCR.

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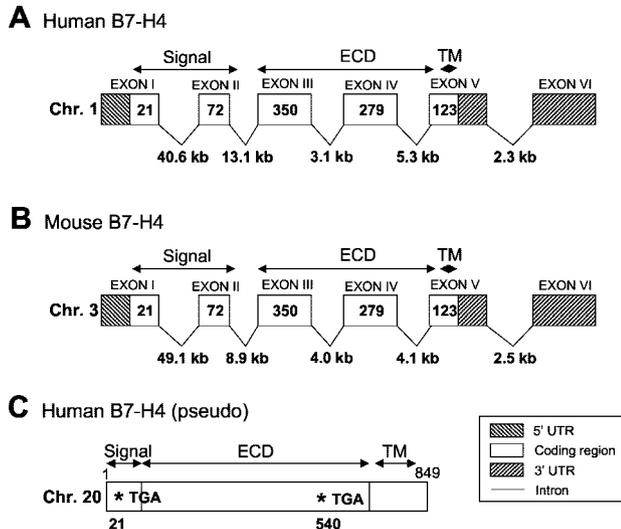
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<sup>4</sup> Abbreviations used in this paper: UTR, untranslated region; h, human.



**FIGURE 1.** Genomic organization of human and mouse *B7-H4* genes. Exons from hB7-H4 (A), mouse *B7-H4* (B), and hB7-H4 pseudogene (pseudo) (C) are shown. The coding region of *B7-H4* (□) and 5' (▨) and 3' (▩) UTR are represented. Numbers in the boxes represent the length of exons in base pairs, and numbers in kilobases mark the length of introns between exons. Asterisks in the exon of hB7-H4 pseudo are stop codons at different sites of the gene.

*Preparation of mAb against hB7-H4*

The method for the generation of mAb to hB7-H4 by immunization of a BALB/c mouse was described previously (10). Three independently generated hybridoma clones, hH4.1, hH4.2, and hH4.3, which secrete mouse mAb (IgG1) against hB7-H4, were selected, and mAb was purified from ascites of BALB/c mouse using protein G affinity column (Pierce, Rockford, IL). Control mouse IgG1 was purchased from Rockland (Gilbertville, PA).

*Immunohistochemistry*

Human cancer and normal tissue samples were obtained from the Pathology Department of the Mayo Clinic with approval of the Institutional Review Board. Frozen tissues were processed as 6- $\mu$ m sections and stained with mAb against *B7-H4* (hH4.3) and with control Ab (mouse IgG1) as described elsewhere (5). Briefly, sections were fixed in cold acetone for 10 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 5 min at room temperature followed by incubation with normal goat sera for 30 min at room temperature. Subsequently, sections were incubated with anti-B7-H4 at 1/100 dilution for 30 min at room temperature. Thereafter, HRP-conjugated anti-mouse IgG was used for secondary Ab, and the staining was developed using a commercial

immunoperoxidase staining kit following the manufacturer's instruction (ABC (avidin/biotin complex); DAKO, Glostrup, Denmark).

*Flow cytometry analysis*

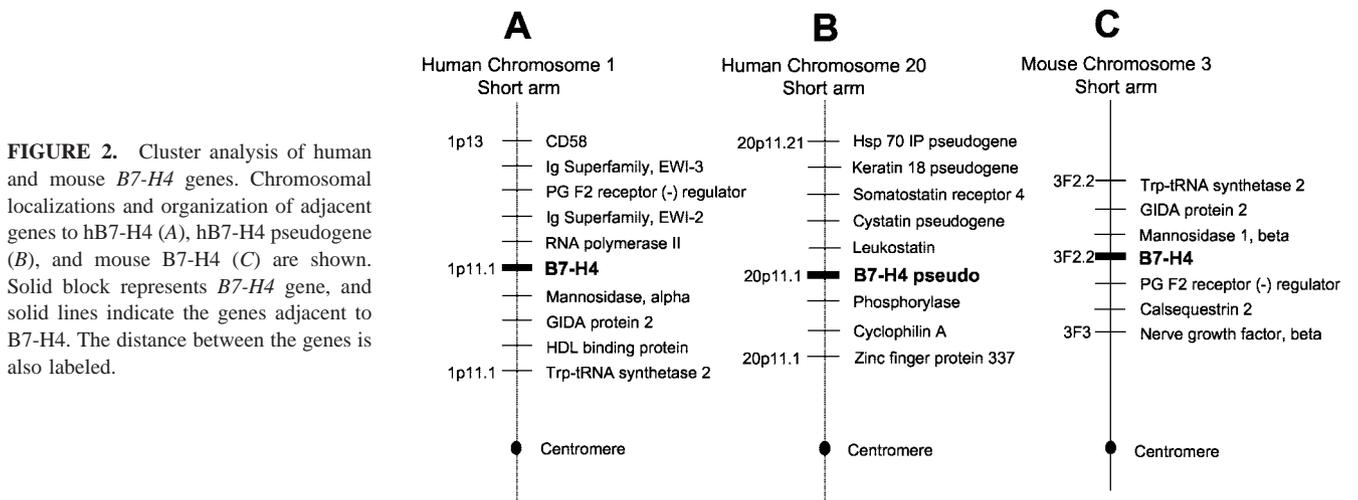
Human ovarian cancer cell lines (11) were generous gifts from Dr. K. R. Kalli (Department of Medicine, Mayo Clinic). Cell lines were grown in  $\alpha$ -MEM Earle's medium containing glutamine, nucleosides, and 20% FBS (Cellgro, Herndon, VA). For flow cytometry analysis of *B7-H4* expression on cell lines, cells were stained with 0.5  $\mu$ g of biotinylated anti-hB7-H4 (hH4.2) mAb or control mouse IgG1, and then with PE-conjugated streptavidin. All staining procedures were done at 4°C for 30 min. FACS results were analyzed with CellQuest software (BD Biosciences, Mountain View, CA) as described (4).

**Results and Discussion**

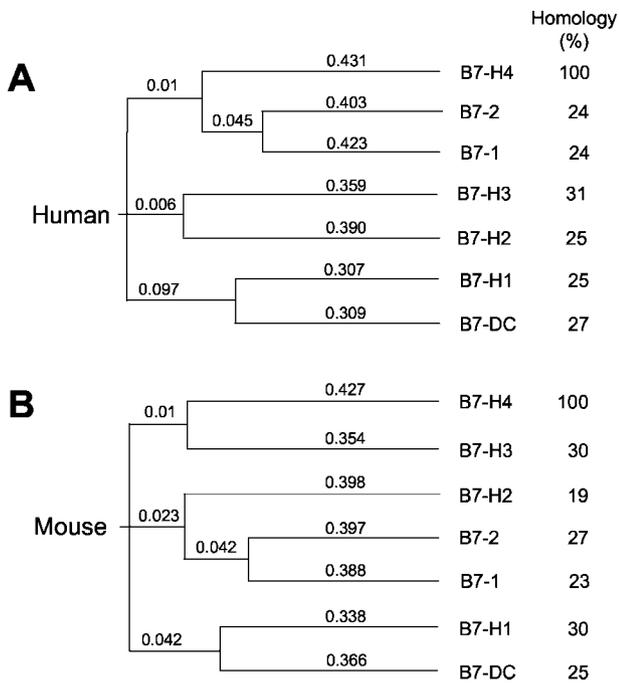
*Genomic organization of human and mouse B7-H4*

BLAST search using NCBI database revealed that the human *B7-H4* gene is located on chromosome 1p11.1. Genomic DNA of *B7-H4* consists of six exons and five introns, on which the coding region of *B7-H4* spans 849 bp (Fig. 1A). Mature protein is encoded by exon III, IV, and part of exon V. Mouse *B7-H4* gene is located on chromosome 3 with six exons and five introns (Fig. 1B). In both genes, exons I and II encode a signal peptide, and IgV-IgC domain comprised of the extracellular region spans through exons III and IV and part of exon V. The transmembrane and intracellular region is located in exon V. 5' and 3' UTRs fall on part of exon I and exons V and VI, respectively (Fig. 1).

However, analysis in both NCBI and Celera databases reveals that the additional hB7-H4 sequence is also located in chromosome 20p11.1. This new sequence contains only a single large exon with up to 94% identity in the nucleotide sequence compared with cDNA of *B7-H4*, and most sequence differences mapping to IgV and IgC domain. Moreover, the sequence contained two stop codons in the coding region at nt 21 and 540 (Fig. 1C). Because nt 21 is located within the signal peptide and nt 540 positions before transmembrane region, these will prevent synthesis of the full length of *B7-H4* protein. Therefore, this alternative *B7-H4* genomic DNA may represent a *B7-H4* pseudogene. This suggests that *B7-H4* protein might be derived from chromosome 1 rather than chromosome 20. Even though deduced amino acid sequences of *B7-H4* from chromosome 20 include the stop codons at signal peptide and IgC domain, we cannot exclude the possibility that *B7-H4* is produced as a truncated form. The functions of this pseudogene are unknown, but the role of the expressed pseudogene in the regulation of mRNA stability of its homologous coding gene has been suggested in a recent report (12).



**FIGURE 2.** Cluster analysis of human and mouse *B7-H4* genes. Chromosomal localizations and organization of adjacent genes to hB7-H4 (A), hB7-H4 pseudogene (B), and mouse *B7-H4* (C) are shown. Solid block represents *B7-H4* gene, and solid lines indicate the genes adjacent to *B7-H4*. The distance between the genes is also labeled.



**FIGURE 3.** Phylogenetic analysis of the B7 family molecules. The sequences of extracellular region of B7 family members of human (A) and mouse (B) were aligned with ClustalW algorithm in MacVector 6.5, and phylogeny was inferred using the Neighbor joining method. The scores at each branch represent the degree of sequence variability between the molecules. Percentage of overall homology of other members to B7-H4 is indicated.

Database analysis also demonstrates that several Ig superfamily genes reside adjacent to B7-H4 including CD58 (LFA-3), Ig superfamily members 2 and 3 (EWI-2, -3) across 1p13 (Fig. 2A), suggesting a potential functional cluster of immunoregulatory genes in this location. However, the majority of these genes are not found in mouse genomic DNA (Fig. 2C). Interestingly, the B7-H4 pseudogene is also found to cluster with multiple other pseudogenes including heat shock protein 70 IP, keratin 18, and cystatin

(Fig. 2B). Phylogenetical analysis by ClustalW algorithm showed that hB7-H4, although it shares 24–31% identity with members in the B7 family, has a closer relationship to CD80 and CD86 among others. B7-H4 shares highest homology at the amino acid level with B7-H3 (human, 31%; mouse, 30%), a molecule with potent costimulatory function on T cells (9), among all B7 family molecules described so far (Fig. 3).

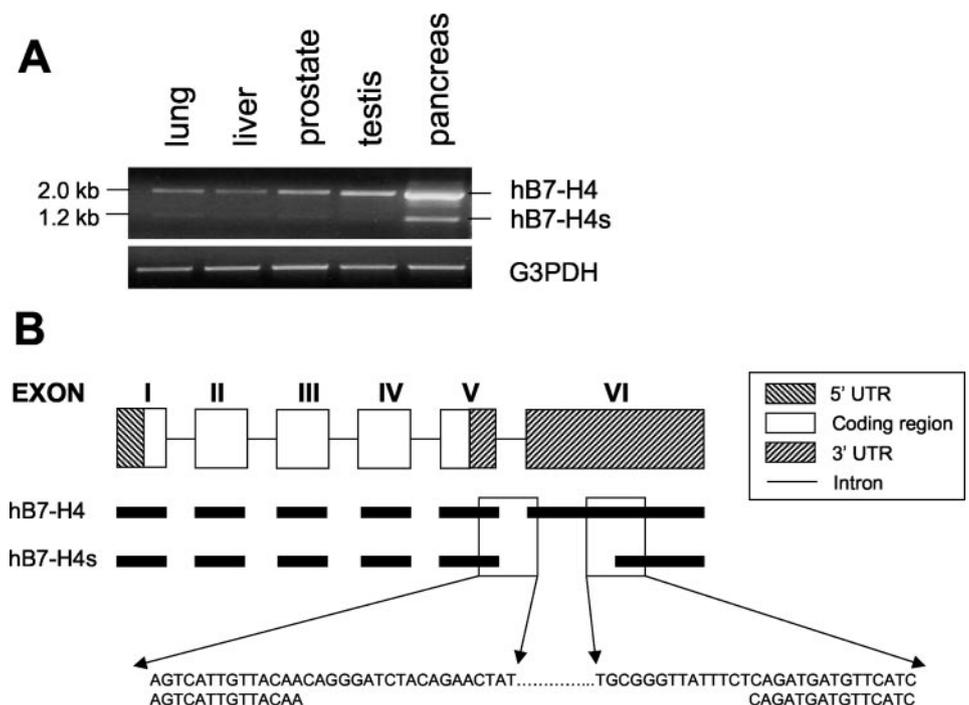
#### B7-H4 transcription

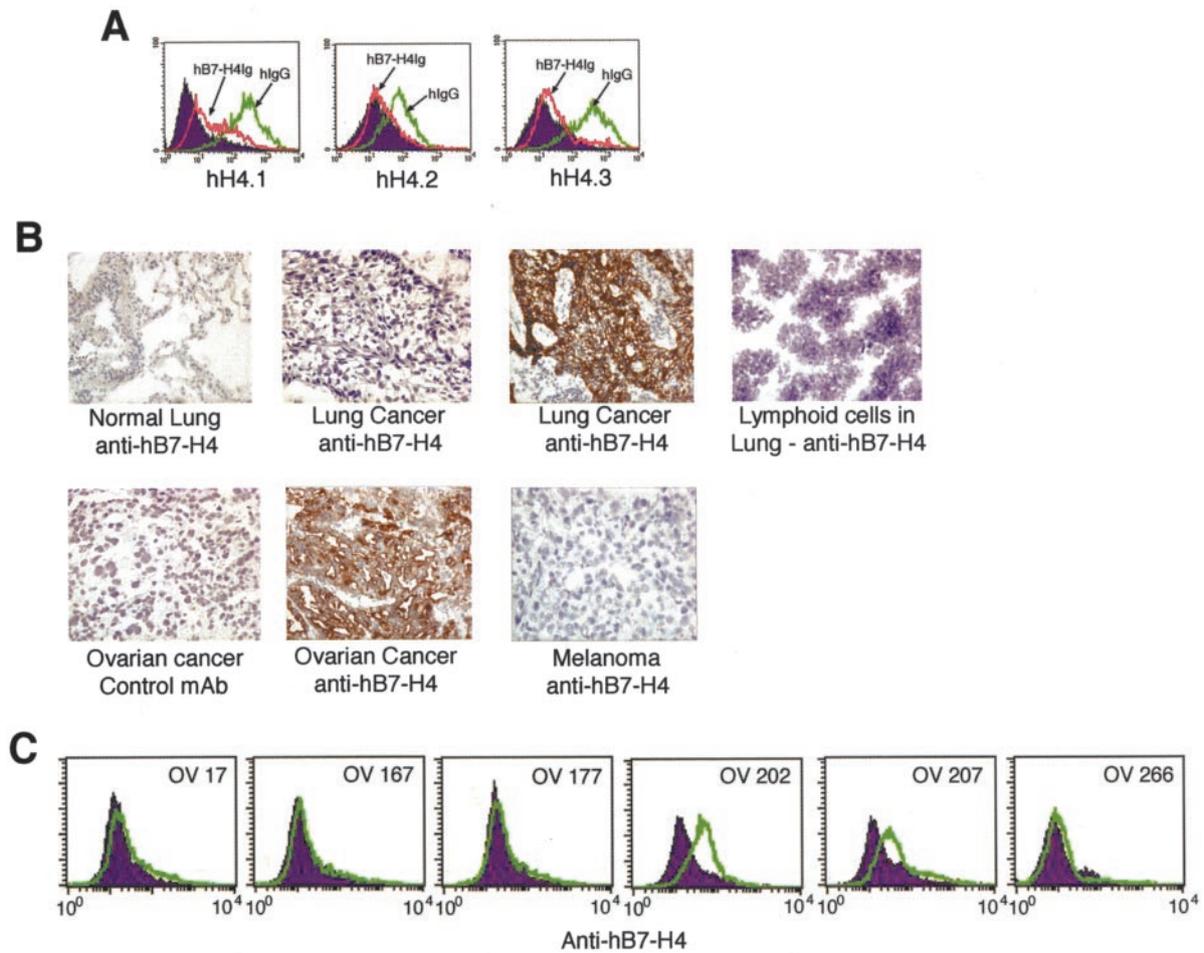
By two primers located in the region next to the putative signal peptide and in the 3' UTR regions of hB7-H4 and by reverse transcription, the B7-H4 mRNA could be detected in many human tissues including placenta, kidney, liver, lung, ovary, testis, and spleen, but not in the brain and heart (8). In addition to a dominant species in ~2.0 kb, PCR analysis revealed an ~800-bp transcript expressed in some tissues (Fig. 4A). The expression of these transcripts was not tissue specific, because most of the tissues demonstrating B7-H4 transcript contained these two different transcripts. The larger transcript represented a major population of messages from most tissues, as shown by the gel electrophoresis. To determine the sequences of the transcripts, we amplified B7-H4 using multiple tissue cDNA panel and the same primers as mentioned above spanning exons I and VII. DNA sequencing analysis indicated that both transcripts shared the complete homology with full-length B7-H4 cDNA corresponding to exons I through V. The smaller transcript was generated by alternative splicing of B7-H4 mRNA where part of exon VI was spliced out (Fig. 4B). Unlike B7-1 (CD80) and B7-2 (CD86) known to have an alternatively spliced variant deleting transmembrane region (13, 14), two different B7-H4 transcripts are predicted to have the identical open reading frame with intact transmembrane domains. It remains to be answered whether this difference in 3' UTR between the transcripts affects the efficiency of translation of the B7-H4.

#### Expression of B7-H4 protein by human cancers

To examine B7-H4 protein expression, we prepared a panel of mouse mAbs against hB7-H4. Three independent hybridomas, hH4.1, hH4.2, and hH4.3, were identified. Flow cytometry analysis

**FIGURE 4.** Identification of hB7-H4 alternative transcripts. A, Expression of hB7-H4 mRNA was demonstrated by the RT-PCR analysis of multiple normal tissue cDNA panels. Full-length transcript (hB7-H4) is ~800 bp longer than shorter version (hB7-H4s) as determined by gel electrophoresis. *G3PDH* gene amplification is included as control for PCR product loading. B, Diagram of B7-H4 transcripts was created by the comparison of sequences of both species. The splicing out of the 5' half of exon VI generated the hB7-H4s transcript. Inserted sequences indicate the comparison of sequences between two species showing the difference produced by alternative splicing.





**FIGURE 5.** Expression of B7-H4 in normal and cancer tissues. *A*, Specificity of mAbs (hH4.1, hH4.2, and hH4.3) against hB7-H4 was determined by flow cytometry analysis using 293T cells expressing hB7-H4 (open) and mock-transfected cells (filled). Each mAb was also tested for competitive binding in the presence of 10  $\mu$ g of hB7-H4Ig. *B*, Immunohistochemistry analysis of snap-frozen specimens of normal human lung tissue, lung cancer, ovary carcinoma and melanoma, and infiltrating lymphoid cells in lung cancer tissues are shown. The mAb hH4.3 was used for analysis. In lung and ovarian cancer samples, B7-H4 immunoreactivity was limited to the tumor plasma membrane and/or cytoplasm. Isotype-matched mouse IgG1 mAb was the control. *C*, Expression of hB7-H4 on ovarian cancer cell lines. The indicated ovarian cancer lines were stained with either mouse IgG1 (filled) or hH4.2 (open) and analyzed by flow cytometry analysis.

demonstrates that these mAb bound to 293T cells expressing hB7-H4 on the surface. The binding could be abrogated by inclusion of soluble hB7-H4Ig fusion protein (Fig. 5A). In ELISA, these mAb did not bind to other members of the hB7 family including B7-1, B7-2, B7-H1, B7-DC, B7-H2, and B7-H3 (data not shown). Our results indicate that these mAb are specific for hB7-H4. Although these mAb were independent clones, it is unknown at this

time whether or not they share the same binding epitopes on B7-H4 molecules.

By immunohistochemistry, we examined the expression of B7-H4 protein on normal human tissues using these mAbs. In contrast to constitutive expression of the B7-H4 mRNA, no staining was found in all normal tissues examined so far, including lung (Fig. 5B), colon, liver, skeletal muscle, kidney, pancreas, small

Table I. Expression of B7-H4 in human cancer tissues

Diagnosis	Specimen Numbers, Positive/Total (%)	Cases with Staining Intensity <sup>a</sup>			
		-	+	++	+++
Lung cancer	5/16 (31)	11	1	1	3
Adenocarcinoma	0/7	7	0	0	0
Squamous cell carcinoma	4/6	2	0	1	3
Large cell carcinoma	1/2	1	1	0	0
Neuroendocrine carcinoma	0/1	1	0	0	0
Ovarian cancer					
Adenocarcinoma	22/26 (85)	4	1	4	17
Melanoma	0/17 (0)	17	0	0	0

<sup>a</sup> Intensity of staining by mAb against B7-H4: -, negative; +, focal expression in 10–40% of cancer tissues; ++, focal expression in 40–80% of cancer tissues; +++, diffuse expression in >80% of cancer tissues.

bowel, breast, and uterus (data not shown). These results suggest that B7-H4 expression is tightly controlled in the translational level in peripheral tissues. In contrast, immunohistochemical analysis demonstrated that B7-H4 was constitutively expressed in freshly isolated ovarian cancer (22 of 26 patients) and lung cancer tissues (5 of 16 patients), but not in all melanoma samples tested so far (0 of 17 patients) (Fig. 5B and Table I). It is of interest that B7-H1, an inhibitory molecule of the B7 family, was also found on some lung and ovarian cancers (5). However, the expression pattern of these two molecules is quite different. For example, B7-H1 was found to express on 22 of 22 melanoma samples, whereas B7-H4 was not detectable in all 17 samples tested so far from the same melanoma patients (Table I). Infiltrating lymphoid cells in lung cancers also did not express B7-H4. B7-H4 expression on tumor cells appears to be very diffuse across the sections in most cases. Microscopically B7-H4 was found in either cytoplasm or plasma membrane of lung and ovarian cancer cells. Constitutive expression of B7-H4 was also found on two of six ovarian cancer cell lines tested so far by flow cytometry analysis (Fig. 5C). Our results thus support an aberrant expression of B7-H4 by human cancers. During the process of publishing this report, Prasad et al. (15) showed that B7S1, which has identical amino acid and nucleic acid sequences with those of B7-H4, could be cleaved by phosphatidylinositol-specific phospholipase C, and this suggests that B7-H4 is a GPI-linked protein. However, we found that phosphatidylinositol-specific phospholipase C treatment did not affect the expression of transfected B7-H4 on EL4 cells while Thy1.2 on the same cells were largely cleaved (G. Zhu and L. Chen, unpublished observation). Our result thus does not support B7-H4 as a GPI-linked protein.

It has been reported that B7-H4 is a potent negative costimulator or coinhibitor for T cell responses upon TCR signaling. Engagement of B7-H4 to a putative receptor on activated T cells could inhibit growth, cytokine secretion, and development of cytotoxicity. In vitro studies demonstrated that B7-H4 inhibits cell cycle progression in the G<sub>0</sub>/G<sub>1</sub> phase (8). It is thus of interest to determine whether the expression of B7-H4 correlates with disease progression on ovarian and lung cancers. Unfortunately, our mAb to B7-H4, as well as mAb to B7-H1, did not bind to paraffin-fixed tissues, which prevents a large-scale retrospective study. Because B7-H4 is not detected in the majority of normal tissues and cells,

constitutive expression of B7-H4 on human cancers may play a role in tumor escape from destruction by the immune system.

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