Review

L1-CAM as a Target for Treatment of Cancer with Monoclonal Antibodies

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Abstract. *L1 cell adhesion molecule (L1-CAM) is a neuronal adhesion molecule which is expressed in many tumor entities. L1-CAM was shown to be involved in proliferation, invasion and metastasis both in vitro and in vivo. L1-CAM is engaged in homophilic interactions and complexes with many other ligands in a context-dependent manner. Activation and modulation of the extracellular signal-related kinase pathway by L1-CAM has been documented. In normal tissues, L1-CAM expression is restricted to nerve bundles and kidney tubules; however, L1-CAM is expressed in many tumor entities and, with the exception of neuroblastoma, L1-CAM expression correlates with poor prognosis. L1-CAM occurs in two isoforms, full-length L1-CAM and a variant in which exons 2 and 27 have been deleted. Preclinical experiments with available monoclonal antibodies are summarized and L1-CAM is analysed as a target for treatment of cancer with monoclonal antibodies.*

In recent years, several monoclonal antibodies have been approved by the FDA for treatment of cancer. Among these are alemtuzumab (Campath) for treatment of chronic lymphocytic leukemia (CLL), bevacizumab (Avastin) for colon, lung cancer, glioblastoma and other types of cancer, cetuximab (Erbitux) for colon and head and neck cancer, panitumumab (Vectibix) for colon cancer, rituximab (Rituxan) for non-Hodgkin's lymphoma and trastuzumab (Herceptin) for breast cancer. In addition, three conjugates have been approved: gemtuzumab (Mylotarg), an antibody-calicheamycin conjugate for acute myelogenous leukemia, and two radioisotope-conjugates of monoclonal antibodies, ibritumomab (Zevalin) and tositumomab (Bexxar) for treatment of NHL. The attractive features of monoclonal

antibodies are a documented increase of therapeutic benefit in comparison to standard of care therapy, especially in combination with chemotherapy, and favorable pharmacokinetic properties which allow administration schedules such as weekly or biweekly injections (1-3). With this class of biological therapeutics, common side-effects have been observed such as skin rashes, diarrhea, nausea, flu-like symptoms, allergic reactions and low blood counts which may lead to infections, bleeding and fever. But also more severe side-effects such as infusion reactions, heart failure and heart attacks have been reported.

Antibodies may interfere with the proliferation of tumor cells *in vitro* and *in vivo* and induce cell-cycle inhibition and apoptosis in tumor cells in which the antigen the antibody is directed against has an oncogenic function. In addition, depending on the isotype and the epitope, immune effector functions such as complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity can be exerted. In most therapeutic regimens antibodies are combined with chemotherapy or other drugs resulting in enhancement of efficacy.

Criteria for selection of transmembrane or membrane-associated targets for antibody-based therapy of cancer are: selectivity of antigen expression in normal *versus* tumor tissue, overexpression in tumors in comparison to matching normal tissues, involvement in tumorigenesis and maintainance of the transformed phenotype. In this context, we have summarized the features of the possible role of the adhesion-molecule L1-CAM for antibody-related therapy of cancer.

General Features of L1-CAM

L1-CAM is a member of the L1 family of adhesion molecules which are members of the immunoglobulin superfamily (IgSF CAMs). The members of the L1 family are L1-CAM (CD171), close homolog of L1-CAM (CHL1), neurofascin and NgCAM-related cell adhesion molecule (NR-CAM) (4-6). Amino acid sequence comparisons are shown in Figure 1. Between human L1-CAM and its paralogs NR-CAM, neurofascin and CHL1,

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an amino acid homology of 40%, 39% and 40%, respectively, has been observed. The conserved exon-intron organization between human L1-CAM and its paralogues as shown in Figure 1 supports the assumption that they are derived from a primordial gene during evolution. They are found on neurons, especially on their axons and glial cells such as Schwann cells. L1-CAM is a neural cell adhesion molecule that is involved in the development of the central nervous system. Its topology and the amino acid sequences are shown in Figures 2 and 3. L1-CAM is composed of 28 exons and 27 introns and the molecular weight of its gene product ranges between 200 and 220 kDa (7). The extracellular domain consists of six Ig-like domains and five fibronectin-like domains (8, 9) and an *N*-glycosylation site is located in the first fibronectin domain. An RGD motif was identified in the first Ig-like domain. The transmembrane domain is located on exon 25 and the carboxyterminal cytoplasmic domain is encoded by exons 26, 27 and 28. Further features of the extracellular domain are homophilic binding and a homology region with FGFR. The cytoplasmic domain contains five potential phospho-serine residues and can interact with the cytoskeleton, second messenger pathways and kinases (9-10). Two exons (2 and 27) are spliced alternatively (7, 10, 11). Figure 4 shows an amino acid alignment of human, rhesus monkey, rat, mouse, chicken, zebrafish and drosophila L1-CAM orthologs. The following percentage homologies have been noted: 99% human *versus* rhesus, 89% *versus* rat, 88% *versus* mouse, 49% *versus* chicken, 41% *versus* zebrafish and 30% *versus* drosophila. The number and location of the Ig-like and fibronectin-like domains are conserved between the species as outlined above. The RGD motif is conserved between the mammalian species shown in Figure 4 (human, rhesus monkey, rat and mouse).

L1-CAM is involved in axon guidance, neural cell migration and differentiation (10) and mutations in the gene cause X-linked neurological disorders known by the acronym CRASH (corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraplegia and hydrocephalus) (11-13). The clinical picture resulting from L1-CAM mutations is extremely variable: more than 70 L1-CAM mutations have been described in all parts of the L1-CAM molecule in CRASH patients (11-13). L1-CAM knock-out mice show hyperplasia of the corticospinal tract and abnormalities of the ventricular system (14, 15). L1-CAM mediates adhesion to different substrates in a context-dependent manner (9). L1-CAM is involved in homophilic binding and was shown to interact with a plethora of adhesion molecules such as axonin-1/TAX-1, contactin, neurocan, neuropilin 1 and integrins such as $\alpha v\beta 3$, $\alpha 5\beta 1$, $\alpha v\beta 1$ and $\alpha v\beta 5$. *Cis* (between molecules in the same cell membrane) and *trans* (between molecules on opposing membranes) interactions have been described (9).

The extracellular domain of L1-CAM contains *N*-linked carbohydrates which can comprise up to 25% of the molecular weight of L1-CAM. Two sites of proteolytic cleavage have been identified in the extracellular domain of L1-CAM. Cleavage at the distal site mediated by the metalloprotease ADAM 10 results in fragments of 200 and 32 kDa (16, 17). Binding interactions of the cytoplasmic region of L1-CAM are reviewed elsewhere (18).

The L1-CAM isoforms are discussed separately in this paper. L1-CAM was shown be involved in multiple proliferation-, anti-apoptosis- and angiogenesis-related pathways as outlined in Figure 5 and in the following parts of this review.

L1-CAM Expression in Tumors and Assessment of L1-CAM as a Predictive Marker

A gene expression data set derived from the Gene Expression Omnibus (GEO) database (GSE 2361) which compares 36 different normal human tissues indicates tissue-restricted expression of L1-CAM with a strong preference for expression in the brain as shown in Figure 6. *In silico* analysis of RNA transcripts for L1-CAM indicated strong overexpression of L1-CAM RNA in different subtypes of ovarian carcinoma (clear cell, endometroid, mucinous and serous) in comparison to normal ovarian tissue as shown in Figure 7A.

In a retrospective study comparing 58 ovarian carcinomas and 72 uterine adenocarcinomas, L1-CAM expression was identified as a marker for prediction of short survival (19). This finding fits with the hypothesis that L1-CAM expression and cleavage could promote dissemination of tumors by facilitating cell migration. The correlation as outlined above is independent of the tumor histotype. Monitoring of soluble L1-CAM during the follow-up period resulted in disease progression and recurrence being indicated before clinical symptoms were noted (19, 20). L1-CAM expression was correlated with disease progression even in stage I endometrioid-type endometrial cases, identifying them as high-risk patients. In an independent study (21) of ovarian serous neoplasms (20 cystadenomas, 14 borderline tumors and 47 carcinomas), L1-CAM immunoreactivity significantly correlated with stage and grade. Sixty three carcinoma patients with low L1-CAM-expressing tumors exhibited a better response to chemotherapy and had a statistically longer progression-free survival. Differential roles of L1-CAM in ovarian carcinoma and ovarian surface epithelium have been proposed (18). It was shown that L1-CAM supports cell cell adhesion and enhances apoptosis in ovarian surface epithelial cells and has no effect on proliferation and invasion in this type of cell, whereas it inhibits adhesion and apoptosis in ovarian carcinoma cell lines (22). L1-CAM in a membrane-bound or soluble form was identified as a protector from apoptosis in ovarian carcinoma cells, whereas RNAi directed against L1-CAM sensitized cells to apoptosis induction. Cisplatin was shown to up-regulate L1-CAM expression in ovarian carcinoma (23).

Figure 1. *Comparison of amino acid sequences of members of the human L1-family. Sequences have been taken from Swiss-Prot (from top: L1CAM, NRCAM, NFASC, and CHL) and annotated according to their gene structure as derived from the human genome. Identical amino acids, start of exons and RGD-motifs are specified by a color code as outlined.*
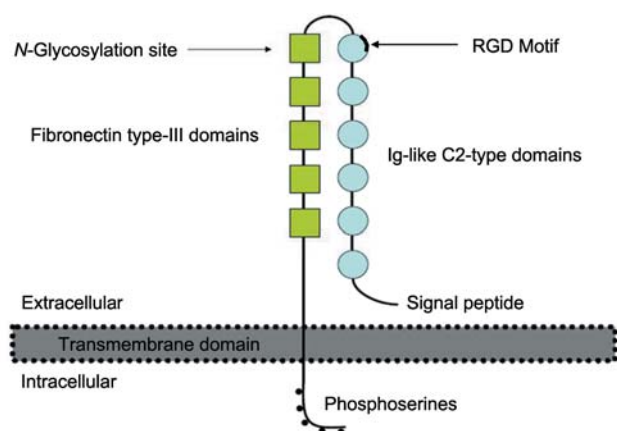
Figure 2. *Schematic topology of human L1-CAM. The fibronectin type III domains are highlighted as green squares and the Ig-like domains are shown as blue circles. The potential N-glycosylation site, the RGD motif of the extracellular domain and the potential intracellular phosphoserine residues of the intracellular domain are also shown.*

Several studies have investigated the role of L1-CAM in progression of malignant melanoma. Comparison of the transcriptional profile of 45 primary melanomas, 18 benign skin nevi and seven normal skin specimen by Affymetrix microarray and confirmation by reverse-transcriptase-polymerase chain reaction (RT-PCR) analysis indicated L1-CAM as a marker to differentiate clinically relevant samples containing benign and malignant melanocytes (24). This finding was supported by a gene expression data set derived from the GEO database (GSE 3189) which points to a strong overexpression of L1-CAM in malignant melanoma compared to nevi and normal skin tissue, as shown in Figure 7B. Immunohistochemical (IHC) analysis of paraffin-embedded specimens of acquired melanocyte nevi, primary cutaneous melanomas and cutaneous and lymph node metastases of malignant melanomas revealed an increase in L1-CAM reactivity in malignant melanomas and metastases as compared to acquired melanocytic nevi. No expression of L1-CAM was detected in melanocytic nevi and melanocytes. Making use of melanoma cells from different stages of progression in monolayer and organotype human skin culture mimicking the pathophysiological environment of cutaneous melanoma, it was found that L1-CAM expression correlates with melanoma progression and $\alpha v \beta 3$ integrin expression (25). Overexpression of L1-CAM in early radial growth phase melanoma cells promotes conversion from radial to vertical growth (25).

Analysis of L1-CAM immunoreactivity in 71 cases of pulmonary neuroendocrine tumors revealed that the percentage of L1-CAM expression increased with the aggressiveness and progression of the tumors, suggesting that L1-CAM immunoreactivity may be a useful diagnostic and prognostic marker in pulmonary neuroendocrine tumors (26). The tumors of 375 patients that underwent surgical treatment for colorectal cancer (CRC) were analyzed retrospectively for L1-CAM expression by IHC. L1-CAM was detected in 48 (13%) of patients. Analysis of L1-CAM expression and survival revealed a significantly worse outcome for L1-CAM-positive patients (27). In an independent study of 138 CRC patients who underwent surgery, L1-CAM expression was investigated in paraffin-embedded blocks of the tumors by tissue microarray analysis. Multivariate analysis revealed that L1-CAM was an independent prognostic marker for patient survival. L1-CAM expression was associated with tumor progression and poor survival in patients with CRC and may be clinically useful as a marker for poor prognosis (27). Additionally L1-CAM is associated with micrometastatic spread and poor outcome in CRC (28). L1-CAM expression was associated with the invasive front of colon tumors (29). Moreover, it was found to be highly expressed in gastrointestinal stromal tumors but not in smooth muscle tumors and desmoid-type fibromatosis (30). This may impact on differential diagnosis.

L1-CAM was found to be specifically expressed in poorly differentiated neuroendocrine pancreatic carcinomas that are known to have the worst prognosis (31). L1-CAM might be a marker for risk prediction in patients with pancreatic neuroendocrine carcinomas. Making use of two antibodies directed to the extracellular and the cytoplasmic domain, it was concluded that L1-CAM is expressed in renal cancer and correlates with metastases in clear cell carcinomas (32). Thirty-one neuroendocrine tumors of the skin (Merkel cell carcinoma) were investigated by IHC for L1-CAM expression (33); L1-CAM expression was detected in most of the tumors and staining was less frequent in metastases and recurrent tumors.

In contrast to tumors in adults where L1-CAM expression is associated with aggressive clinical behavior, expression of L1-CAM was correlated with favorable outcome in pediatric neuroblastomas (34) in a study in which L1-CAM expression was assessed on a tissue microarray with 66 surgically removed neuroblastomas by IHC and RT-PCR. The molecular basis for these findings have not yet been resolved.

Making use of monoclonal antibodies L1-11A and L1-14.10 revealed expression of L1-CAM in tumors of the female genital tract such as adenocarcinomas of the cervix and fallopian tubes, ovarian and endometrial carcinomas. Non-gynecological tumors expressing L1-CAM comprised malignant melanoma, colon cancer, clear-cell carcinomas of the urinary bladder, pheochromocytoma, small cell lung carcinoma, gastrointestinal tract carcinomas, gastrointestinal carcinoids, renal clear cell carcinomas, prostate adenocarcinomas and mesotheliomas (35). Further aspects of L1-CAM expression in cancer tissues have been reviewed recently (36).

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   1   MVVALRYVWP LLLCSPCLLI QIPEEYEGHH VMEPPVITEQ SPRRLVVFPT
  51   DDISLKCEAS GKPEVQFRWT RDGVHFKPKE ELGVTVYQSP HSGSFTITGN
 101   NSNFAQRFQG IYRCFASNKL GTAMSHEIRL MAEGAPKWPK ETVKPVEVEE
 151   GESVVLPCNP PPSAEPLRIY WMNSKILHIK QDERVTMGQN GNLYFANVLT
 201   SDNHSDYICH AHFPGTRTII QKEPIDLRVK ATNSMIDRKP RLLFPTNSSS
 251   HLVALQGQPL VLECIAEGFP TPTIKWLRPS GPMPADRVTY QNHNKTLQLL
 301   KVGEEDDGEY RCLAENSLGS ARHAYYVTVE AAPYWLHKPQ SHLYGPGETA
 351   RLDCQVQGRP QPEVTWRING IPVEELAKDQ KYRIQRGALI LSNVQPSDTM
 401   VTQCEARNRH GLLLANAYIY VVQLPAKILT ADNQTYMAVQ GSTAYLLCKA
 451   FGAPVPSVQW LDEDGTTVLQ DERFFPYANG TLGIRDLQAN DTGRYFCLAA
 501   NDQNNVTIMA NLKVKDATQI TQGPRSTIEK KGSRVTFTCQ ASFDPSLQPS
 551   ITWRGDGRDL QELGDSDKYF IEDGRLVIHS LDYSDQGNYS CVASTELDVV
 601   ESRAQLLVVG SPGPVPRLVL SDLHLLTQSQ VRVSWSPAED HNAPIEKYDI
 651   EFEDKEMAPE KWYSLGKVPG NQTSTTLKLS PYVHYTFRVT AINKYGPGEP
 701   SPVSETVVTP EAAPEKNPVD VKGEGNETTN MVITWKPLRW MDWNAPQVQY
 751   RVQWRPQGTR GPWQEQIVSD PFLVVSNTST FVPYEIKVQA VNSQGKGPEP
 801   QVTIGYSGED YPQAIPELEG IEILNSSAVL VKWRPVDLAQ VKGHLRGYNV
 851   TYWREGSQRK HSKRHIHKDH VVVPANTTSV ILSGLRPYSS YHLEVQAFNG
 901   RGSGPASEFT FSTPEGVPGH PEALHLECQS NTSLLLRWQP PLSHNGVLTG
 951   YVLSYHPLDE GGKGQLSFNL RDPELRTHNL TDLSPHLRYR FQLQATTKEG
1001   PGEAIVREGG TMALSGISDF GNISATAGEN YSVVSWVPKE GQCNFRFHIL
1051   FKALGEEKGG ASLSPQYVSY NQSSYTQWDL QPDTDYEIHL FKERMFRHQM
1101   AVKTNGTGRV RLPPAGFATE GWFIGFVSAI ILLLLVLLIL CFIKRSKGGK
1151   YSVKDKEDTQ VDSEARPMKD ETFGEYRSLE SDNEEKAFGS SQPSLNGDIK
1201   PLGSDDSLAD YGGSVDVQFN EDGSFIGQYS GKKEKEAAGG NDSSGATSPI
1251   NPAVALE
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- Signal peptide
- Ig-like C2-type domains
  - RGD Motif
- Fibronectin type-III domains
  - *N*-Glycosylation site
- Transmembrane domain
- Phosphoserine
- Begin of exon
- Exons 2 and 27

Figure 3. *Amino acid sequence, exon-intron organization and topology of human L1-CAM. The signal peptide, Ig-like domains, fibronectin-like domains, RGD motif and potential N-glycosylation site, transmembrane domain, potential phosphoserine residues, start of exons and exons 2 and 27 are shown by an appropriate color code.*

Figure 4. *continued*

L1-CAM Signaling and Cancer

L1-CAM and ADAM10 expression were shown to confer metastatic capacity of CRC cells to the liver and it was found that genes induced by L1-CAM in CRC cells are expressed at a higher level in tumor tissue than in normal tissue based on analysis of a large set of human CRC and normal tissue samples (36). L1-CAM was shown to be a target of β-catenin-Wnt signaling. Expression of L1-CAM confers cell motility, invasion and tumorigenesis in fibroblasts and colon cancer cells (16, 29, 36). In colorectal tumor tissue, L1-CAM was exclusively localized at the invasive front of the tumor tissue that expresses nuclear β-catenin together with ADAM10 that is involved in cleavage and shedding of the L1-CAM extracellular domain (29). Homophilic and heterophilic L1-CAM binding and concomitant signaling has been shown to promote cell motility. L1-CAM induces and maintains a motile and invasive phenotype by inducing transcription of corresponding genes. In the presence of serum or platelet-derived growth factor, L1-CAM was shown to stimulate the extracellular signal-related kinase (ERK) pathway. Activation of this pathway leads to expression of motility- and invasion-related gene products such as β3 integrin subunit, small GTPases and cysteine proteases cathepsin-L and -B (37-40). In the context of a genetic screening, L1-CAM together with cell surface targets IGF2R and SCL31A1 were identified as survival factors which protect tumor cells such as HCT 116 colon carcinoma cells
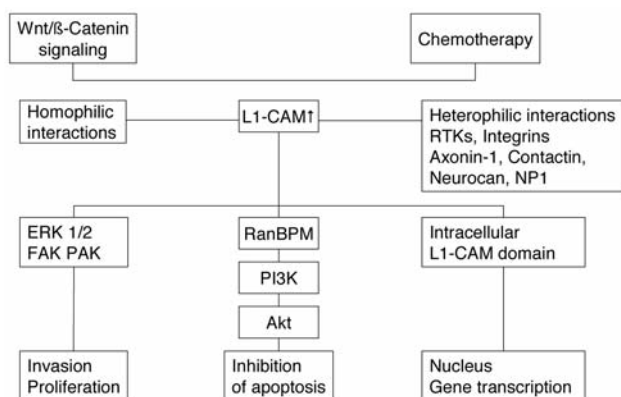
ii

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                                                                        700
HUMAN       LGDSDKYF.I EDGRLVIHSL DYSDQGNYSC VASTELDVVE SRAQLLVVGS PGPVPRLVLS DLHLLTQSQV RVSWSPAEDH NAPIEKYDIE FEDKEMAPEK 661
RHESUS      LGDSDKYF.I EDGRLVIHSL DYSDQGNYSC VASTELDVVE SRAQLLVVGS PGPVPRLVLS DLHLLTQSQV RVSWSPAEDH NAPIEKYDIE FEDKEMAPEK 661
RAT         RGDSDKYF.I EDGQLVIKSL DYSDQGDYSC VASTELDEVE SRAQLLVVGS PGPVPHLELS DRHLLKQSQV HLSWSPAEDH NSPIEKYDIE FEDKEMAPEK 660
MOUSE       RGDSDKYF.I EDGKLVIQSL DYSDQGNYSC VASTELDEVE SRAQLLVVGS PGPVPHLELS DRHLLKQSQV HLSWSPAEDH NSPIEKYDIE FEDKEMAPEK 660
CHICK       ..DDPRYS.V A.AEMTVSNV DYGDEGTIQC RASTPLDSAE AEAQLRVVGR P.PSRDLQVM E...VDEHRV RLSWTPGDDH NSPIEKFVVE EEEEREDLQR 647
ZEBRAFISH   SSDSDKYM.I DESTLIIPEV EEEDEGIYTC EVITTLDMAK ASGSITIVDR PDPPSLIQMT E...PKERRV TLSWTPGADH NSPVLEYLIB FEETGSDRKE 671
DROSOPHILA  FEAQPRFVKT NDNSLTIAKT MELDSGEYTC VARTRLDEAT ARANLIVQDV PNAPKLTGIT ....CQADKA EIHWEQQGDN RSPILHYTIQ F.NTSFTPAS 659
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HUMAN       WYSL.GKVPG NQTSTTLKLS PYVHYTFRVT AINKYGPGEP SPVSETVVTP EAFPEKNPVD VKGEGNETTN MVITWKPLRW MDWNAPQVQY RVQWRPQGTR 760
RHESUS      WYSL.GKVPG NQTSTTLKLS PYVHYTFRVT AINKYGPGEP SPVSETVVTP EAFPEKNPVD VKGEGNETTN MVITWKPLRW MDWNAPQVQY RVQWRPQGTR 760
RAT         WFSL.GKVPG NQTSTTLKLS PYVHYTFRVT AINKYGPGEP SPVSETVVTP EAFPEKNPVD VRGEGNETNN MVITWKPLRW MDWNAPQIQY RVQWRPLGKQ 759
MOUSE       WFSL.GKVPG NQTSTTLKLS PYVHYTFRVT AINKYGPGEP SPVSESVVTP EAFPEKNPVD VRGEGNETNN MVITWKPLRW MDWNAPQIQY RVQWRPGKQ 759
CHICK       GPGA.ADVPG QPWTPPLPLS PYGRFPPFRVV AVNAYGRGEH HAPSAPIETP PAFPERNPGG VHGEGNETGN LVITWEPLPP QAWNAPWARY RVQWRPLEEP 746
ZEBRAFISH   WEEL.SRVSG SSQRATLPLK PFISYRPRVI SINEIGKSDP SMVTEAYSTP AAFPDSNPEG VRSDSDEPGT LVITWKEMDR RSFNGPDFHY KVMWRKVLGS 770
DROSOPHILA  WDAAYEKVPN TDSSFVVQMS PWANYTFRVI AFNKIGASPP SAHSDSQTTQ PDVPFRNPDN VVGQGTEPNN LVISWTPMPE IEHNAPNFHY YVSWKRDIPA 759
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HUMAN       G........P WQBQIVSDPF LVVSNTSTFV PYEIKVQAVN SQGKGP.EPQ VTIGYSGEDY PQAIPELEGI .EILNSSAVL VKWR.PVDLA QVKGHLRGYN 849
RHESUS      G........P WQBQIVSDPF LVVSNTSTFV PYEIKVQAVN SQGKGP.EPQ VTIGYSGEDY PQAIPELEGI .EILNSSAVL VKWR.PVDLA QVKGHLRGYN 849
RAT         E........T WKBQTVSDPF LVVSNTSTFV PYEIKVQAVN NQGKGP.EPQ VTIGYSGEDY PQVSPELEDI .TIFNSSTVL VRWR.PVDLA QVKGHLKGYN 848
MOUSE       E........T WRKQTVSDPF LVVSNTSTFV PYEIKVQAVN NQGKGP.EPQ VTIGYSGEDY PQVSPELEDI .TIFNSSTVL VRWR.PVDLA QVKGHLKGYN 848
CHICK       GGGGPSGGFP WAESTVDAPP VVVGGLPPFS PPQIRVQAVN GAGKGP.EAT PGVGHSGEDL PLVYPENVGV .ELLNSSTVR VRWTLGGGPK ELRGRLRGFR 844
ZEBRAFISH   GP.......S WHTKDITSPP FVVTDVGNFS AFDIKVQAVN EMGEGP.EPK SIIGYSGEDY PLEAPLDVGL .ILINSTAIK VTWA.AVNRE TVRGRLQGYK 860
DROSOPHILA  AAWENNNIFD WRQNNI.... .VIADQPTFV KYLIKVVAIN DRGESNVAAE EVVGYSGEDR PLDAPTNFTM RQITSSTSGY MAWT.PVSEE SVRGHFKGYK 853
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HUMAN       VTYWREGSQR KHSKRHIHKD HVVVP..... .......... ...ANTTSVI LSGLRPYSSY HLEVQAFNGR GSGPASE.FT FSTPBGVPGH PEALHLECQS 930
RHESUS      VTYWREGSQR KHSKRHIHKD HVVVP..... .......... ...ANTTSVI LSGLRPYSSY HLEVQAFNGR GLGPASE.FT FSTPBGVPGH PEALHLECQS 930
RAT         VTYWWKGSQR KHSKRHVHKS HMVVP..... .......... ...ANTTSAI LSGLRPYSSY HVEVQAFNGR GLGPASE.WT FSTPBGVPGH PEALHLECQS 929
MOUSE       VTYWWKGSQR KHSKRHIHKS HIVVP..... .......... ...ANTTSAI LSGLRPYSSY HLEVQAFNGR GLGPASE.WT FSTPBGVPGH PEALHLECQS 929
CHICK       VLYWRLGWVG ERSRRQAPPD PPQIPQSPAE DPPPFPPPVAL TVGGDARGAL LGGLRPWSRY QLRVLVFNGR GDGPFSEPIA PETPBGVPGP PEELRVERLD 944
ZEBRAFISH   IYLTYLGPKG QHLRREPKTT FVVTTD..... .......... ...SNEEKKV LGDLQPFSHY NLAVSVFNYK GEGPQSESIL FFIPBGVPGP PSSLMLDSPS 943
DROSOPHILA  IQTWTEN.BG EBGLREIHVK .......... .......... ...GDTHNAL VTQFKPDSKN YARILAYNGR FNGPPSAVID FDIPBGVPSP VQGLDAYPLG 929
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HUMAN       NTSLLLRWQP PLSHNGVLTG YVLSYHPLDE ...GGKGQLS FNLRDPELRT HNLTDLSPHL RYRFQLQATT KE.GPGEAIV REGGIMAL.. ...SGISDFG 1021
RHESUS      NTSLLLRWQP PLSHNGVLTG YVLSYHPLDE ...GGKGQLS FNLRDPELRT HNLTDLSPHL RYRFQLQATT KE.GPGEAIV REGGIMAL.. ...SGISDFG 1021
RAT         DTSLLLHWQP PLSHNGVLTG YLLSYHPLDE ...ESKEQLF FNLSDPELRT HNLTNLNPDL QYRFQLQATT HQ.GPGEAIV REGGIMAL.. ...FGKPDFG 1020
MOUSE       DTSLLLHWQP PLSHNGVLTG YLLSYHPVEG ...ESKEQLF FNLSDPELRT HNLTNLNPDL QYRFQLQATT QQGGPGEAIV REGGIMAL.. ...FGKPDFG 1021
CHICK       DTALSVVERR TFKRS..ITG YVLRYQQVEP ...GSALPGG SVLRDPQC.. .DLRGLNARS RYRLALFSTP RE.RPALQTV ..GSIKPB.. ...FPSPLWS 1028
ZEBRAFISH   ETEMTVHWTP PNQPNGNLIG YILQHQQLDN ...DASLMQV VKISDPTASH YKLK.LDPHS RYRFYLRGYT AI.GEGLPII KDGAITLD.. ...FVPP..S 1031
DROSOPHILA  SSAFMLHWKK PLYPNGKLTG YKIYYEEVKE SYVGERREYD PHITDPRVTR MKMAGLKPNS KYRISITATT KM.GEGSEHY IEKTTLDKAV NVAFATPSFS 1028
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HUMAN       NISATAGENY ..SVVSW.VP KEGQ..CNFR PHILFKALGE EKGGASL..S PQYVSYNQSS YTQWDLQPDT DYBIHLF... ..KERMFRHQ MAVKTNGT.G 1108
RHESUS      NISATAGENY ..SVVSW.VP KEGQ..CNFR PHILFKALGE EKGGASL..S PQYVSYNQSS YTQWDLQPDT DYBIHLF... ..KERMFRHQ MAVKTNGT.G 1108
RAT         NISVTAGENY ..SVVSW.VP REGQ..CNFR PHILFKALPE GKVSPDHQFQ PQYVSYNQSS YTQWDLQPDT KYBIHLM... ..REKVLLHH LAVKTNGT.G 1109
MOUSE       NISATAGENY ..SVVSW.VP RKGQ..CNFR PHILFKALPE GKVSPDHQFQ PQYVSYNQSS YTQWNLQPDT KYBIHLI... ..KEKVLLHH LDVKTNGT.G 1110
CHICK       RFGVGGRGGF HGAAVEFGAA QEDD..VEFE VQFMNKSTDE PWRTSGR... ...ANSSLRR YRLEGLRPGT AYRVQFVGRN RSGENVAFWE SEVQTNGT.. 1118
ZEBRAFISH   DISISTGETS ..VNLTW.VS KERQRNVEFF IHYLRKDGPD KWEKSEK... ...VNSTQSF YTLHGLQPGS QYRLKI..... ..DFNNTKWE QBIQTAGA. 1114
DROSOPHILA  WBQLPSDNGL AKFRINWLPS TEGHPGTHF. FTMHRIKGET QWIRENE... ...EKNSDY QEVGGLDPET AYEFRVVSVD G.HFNTESAT QBIDTNTVEG 1119
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iii

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                                                                        1300
HUMAN       RVRLPPAG.F ATEGWFIGFV SAIILLLLVL LILCFIKRSK GGKYSVKDKE DTQVDSEARP MKDETFGEYR SLESDNEE.K APGSSQPSLN GDIK....PL 1202
RHESUS      RVRLPPAG.F ATEGWFIGFV SAIILLLLVL LILCFIKRSK GGKYSVKDKE DTQVDSEARP MKDETFGEYR SLESDNEE.K APGSSQPSLN GDIK....PL 1202
RAT         PVRVSTTGSF ASEGWFIAFV SAIILLLLIL LILCFIKRSK GGKYSVKDKE DTQVDSEARP MKDETFGEYR SLESDNEE.K APGSSQPSLN GDIK....PL 1204
MOUSE       PVRVSTTGSF ASEGWFIAFV SAIILLLLIL LILCFIKRSK GGKYSVKDKE DTQVDSEARP MKDETFGEYR SLESDNEE.K APGSSQPSLN GDIK....PL 1205
CHICK       VVPQPGGG.V CTEGWFIAPV SSVVLLLLIL LILCFIKRSK DTQVDSEARP MKDETFGEYR SLESEARKGS ASGSGAGSGV GSPGRGPCAA 1217
ZEBRAFISH   RVMEVKSG.F VTEGWFIGLI SALVLLLLVL LILCFIKRSK GGKYSVKEKE EGQIDSEARP MNNEAFGEYR SLESDNEE.. KRTASQPSLC EDSK.....L 1206
DROSOPHILA  PIMVANET.V ANEGWFIGMM LALAFIIILF IIICIIRRNR GGKYDVHDRE ...LANGRRD YPEE..GGFH EYSQPLDN.K SAGRQSVSSA NKPG....VE 1208
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                                                                        1396
HUMAN       GSDDSLADYG GSVDVQFNE. .DGSFIGQYS GKKKEKEA... .......... .....AGGND SSGATSPI.. .......NPA VALE...... ...... 1257
RHESUS      GSDDSLADYG GSVDVQFNE. .DGSFIGQYS GKKKEKEA... .......... .....AGGND SSGATSPI.. .......NPA VALE...... ...... 1257
RAT         GSDDSLADYG GSVDVQFNE. .DGSFIGQYS GKKKEKEA... .......... .....AGGND SSGATSPI.. .......NPA VALE...... ...... 1259
MOUSE       GSDDSLADYG GSVDVQFNE. .DGSFIGQYS GKKKEKEA... .......... .....AGGND SSGATSPI.. .......NPA VALE...... ...... 1260
CHICK       GSEDSLAGYG GSGDVQFNE. .DGSFIGQYR G.....P... .......... .....GAGPG SSGPASPC.. ........AG PPLD...... ...... 1266
ZEBRAFISH   CTDDGLDDYA NSNSVQTEVI MDESLASQSS GVRDV.P... .......... .....DAETQ ESSPLNPATA ISHHGLPNSA ALLD...... ...... 1271
DROSOPHILA  SDTDSMABYG DGDTGQFTB. .DGSFIGQYV PGKLQPPVSP QPLNNSAAAH QAAPTAGGSG AGSAAAAGA SGGASSAGGA AASNGGAAAG AVATYV 1302
```

- Residues in agreement with consensus
- RGD Motif
- Yellow box: Signal peptide
- Blue boxes: Ig-like C2-type domains
- Green boxes: Fibronectin type-III domains
- Gray box: Transmembrane domain
- **Exons 2 and 27 (bold)**

Figure 4. *Comparative amino acid sequences of L1-CAMs from different species. Sequences have been taken from UniProt and RefSeq (from top: SW:L1CAM_HUMAN, RSP:XP_001087861, SW:L1CAM_RAT, SW: L1CAM_MOUSE, SW:NGCA_CHICK, TR:B3DGN9_DANRE, SW:NRG_ DROME) and annotated according to L1CAM_HUMAN. Identical amino acids, Ig-like domains, transmembrane domain, exon 2 and 27 and RGD motifs are boxed and/or highlighted by specified color code.*

Figure 5. *L1-CAM-mediated interactions and involvement in signaling pathways. L1-CAM is up-regulated by chemotherapy and Wnt/β-catenin signaling, interacts with membrane proteins via homo- and heterophilic interactions and mediates invasion, proliferation, anti-apoptotic and angiogenesis-related signaling effects.*

from apoptosis (41). These findings were supported by the fact that RNAi directed against L1-CAM was shown to induce apoptosis in HCT 116 cells.

The role of L1-CAM in cell migration is probably cell type and context dependent. L1-CAM-dependent up-regulation of $\alpha v \beta 3$ integrin involving activation of ERK was described. However, it was shown that L1-CAM and $\alpha v \beta 3$ are not co-expressed in ovarian carcinoma. Overexpression of L1-CAM did not up-regulate $\alpha v \beta 3$ in ovarian carcinoma cells, but was able to do so in HEK 293 cells (42). The binding of L1-CAM on ovarian carcinoma cell lines to neuropilin-1 on mesothelial cells which form the lining of the peritoneum was demonstrated. Likewise, soluble L1-CAM also binds to neuropilin-1. This interaction may contribute to growth of ovarian carcinomas and to reciprocal signaling between mesothelial cells and tumors (43).

In line with the function of L1-CAM as a mediator of the epithelial-mesenchymal transition effects are findings in breast carcinoma MCF-7 cells which express the non-neuronal isoform of L1-CAM. Knock-down of L1-CAM revealed that L1-CAM expression leads to disruption of adherens junctions and increases β-catenin transcriptional activity. Expression of the non-neuronal isoform of L1-CAM was found in 16 out of 17 tumor cell lines originating from different tumor types (44).

The *C*-terminal fragment of L1-CAM is translocated to the nucleus and is involved in L1-CAM-dependent gene regulation (37). Full-length L1-CAM has to undergo sequential cleavage by ADAM 10 and presenilin/γ secretase in order to reach the nucleus. It was shown that the RGD binding site located in the sixth Ig domain of L1-CAM is important for nuclear signaling (40) The corresponding mutant protein was unable to translocate to the nucleus. Shedding of L1-CAM and its

physiological consequences has been investigated (36). As outlined, soluble L1-CAM is produced by metalloproteinase-mediated ectodomain shedding of L1-CAM. In addition, it was shown that hepatocyte growth factor (HGF) mediates release of a 180 kDa form of L1-CAM into the media of renal carcinoma cells in a dose-dependent manner (45). Making use of L1-CAM mutants, it was demonstrated that the cytoplasmic domain of L1-CAM regulates basal shedding and association with the cytoskeleton through the ankyrin binding site is involved in shedding. Constitutive cleavage of L1-CAM can occur in exosomes as shown in ovarian carcinoma cell lines (46). Constitutive cleavage is mediated by ADAM 10, a disintegrin and metalloproteinase 10. Exosomes are continuously released from the cells and can be found in the ascites fluid and serum of ovarian cancer patients. It was shown that soluble L1-CAM (sLI-CAM) is a mediator of angiogenesis probably due to ligation of integrins based on interaction with the RGD motif (47). The angiogenic activity of sL1-CAM could be abolished by a chimeric antibody directed against L1-CAM, chCE7. sL1-CAM induced proliferation, matrigel invasion and tube formation of bovine aortic endothelial cells and revealed proangiogenic activity in the chick chorioallantoic membrane assay. sL1-CAM is a ligand for several integrins and can be deposited in the extracellular matrix.

Another important issue of L1-CAM is its involvement in chemoresistance (apoptosis resistance). It was found by making use of L1-transfectants that ovarian carcinoma cells expressing L1-CAM are more resistant to apoptosis (48). Treatment with apoptotic stimuli up-regulated the anti-apoptotic molecule Bcl-2 to a greater extent in HEK 293 cells expressing L1-CAM. In HEK-293 cells, L1-CAM mediates ERK, FAK and PAK phosphorylation. Selection of m130 ovarian carcinoma or SW 207 colon carcinoma cells with cisplatin leads to up-regulated expression of L1-CAM. In the ovarian carcinoma cell line OVMZ, knock-down of L1-CAM by RNAi sensitized cells to apoptosis induction. Similar findings of drug-induced expression of L1-CAM conferring anti-apoptotic protection and chemoresistance were described for pancreatic ductal adenocarcinoma cells (PT 45-P1 res cells) (49). L1-CAM knock-down by RNAi in this cell line led to an increase of anticancer drug-induced caspase activation. Conversely, overexpression of L1-CAM in PT 45-P1 cells conferred anti-apoptotic protection against anticancer drug treatment. IHC analysis revealed expression of L1-CAM in 80% of pancreatic adenocarcinomas.

L1-CAM Splice Variants

L1-CAM is normally found in neural tissue, whereas non-neural cells including cancer cells, predominantly express the variant lacking exons 2 and 27 (50-55). The biological functions of exons 2 and 27 have been studied in the nervous system. Exon 2 is important for homophilic L1-L1 binding
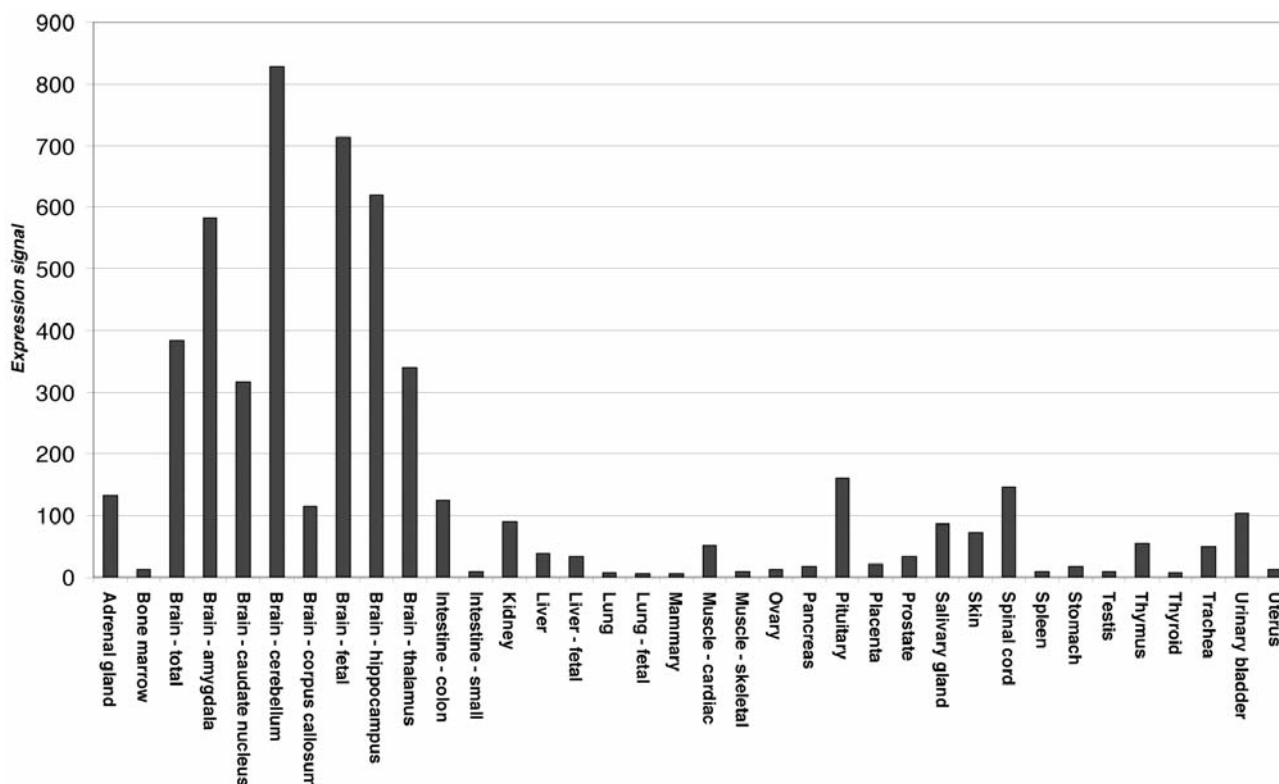
Figure 6. *RNA-based expression of L1-CAM in normal human tissues. Expression of L1-CAM in normal tissues was determined by microarray analysis. Each expression value correlates to the amount of mRNA measured. A value greater than 100 indicates expression in the respective tissue. Data were derived from the Gene Expression Omnibus (GEO) database (data set GSE2361). In this study, pooled RNA samples from 2 to 84 donors were used.*

in vitro and is required for optimal binding to heterophilic ligands (52, 56). RSLE sequence containing L1-CAM encoded by exon 27 is internalized 2-3 times faster than L1Δ(RSLE) (57). RSLE-dependent endocytosis serves as a mechanism to regulate the amount of surface L1-CAM, thereby controlling neurite branching and cell adhesion (57-59). It was found that ovarian carcinoma cell lines BW and GG predominantly express L1-CAMΔ (2, 27) *in vitro* (50). Unfortunately, L1-CAM isotype-specific monoclonal antibodies for IHC detection of the variants are presently not available. L1-CAM induces activation of the ERK pathway and activation of this pathway by L1-CAM was shown to require endocytosis of L1-CAM mediated by exon 27 (60, 61). It was shown that isoform-expressing cells containing the RSLE sequence encoded by exon 27 migrate much better on L1-CAM substrates (61). Based on findings with extracellular matrix substrates for migration, it was shown that cell migration is based on rapid recycling of β1-integrins (61). The functional significance of L1-CAM splice variants in cancer is under investigation. It has been shown that different splice variants of one gene may have different or even opposing functions in the biology of cancer (63, 64).

Antibodies Directed against L1-CAM

Anti-neuroblastoma antibody chCE7 was shown to bind to L1-CAM on renal carcinoma cells and is internalized by human neuroblastoma cells (64, 65). It was found that chCE7 binds near to the sixth Ig-like domain of human L1-CAM, which contains a single RGD sequence. L1-CAM antibodies chCE7 and L1-11A inhibit proliferation of ovarian carcinoma SKOV3ip cells and other L1-CAM-positive tumor cells (renal, neuroblastoma, colon) (66, 67). For two other cell lines, cross-linking with a secondary antibody was necessary for significant inhibition of proliferation by L1-11A, but not by chCE7. Biweekly treatment of ovarian carcinoma-bearing mice with L1-11A led to a dose-dependent and significant reduction of tumor burden (up to 63.5%) and ascites formation (up to 75%) (68). Genistein potentiates the anti-proliferative and pro-apoptotic effects of chCE7 in SKOV3ip cells (69). This was reflected by reduction of the sensitivity of p44/42 (ERK1, 2) kinase, src and Akt to stimulation with serum, EGF and HGF. L1-CAM augments tumor growth and invasion due to induction of ERK-dependent genes and this effect can be inhibited by monoclonal antibodies directed against L1-CAM.
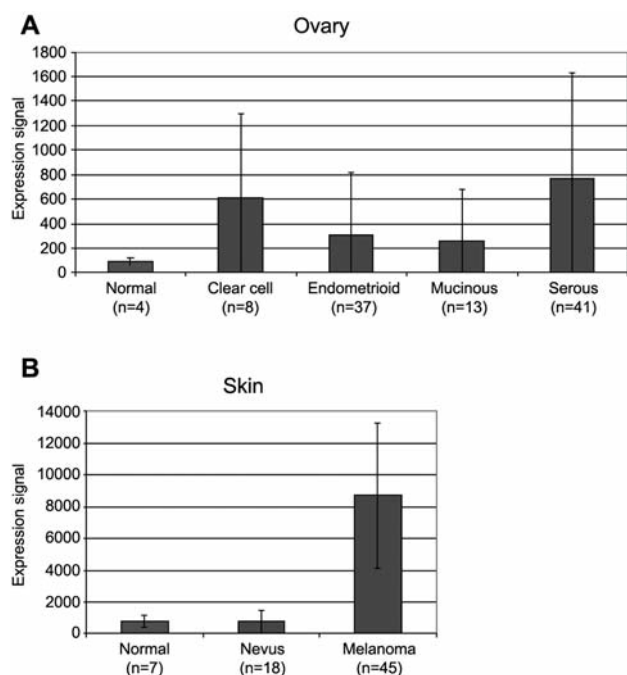
Figure 7. *RNA-based L1-CAM expression in ovarian carcinoma and melanoma in comparison to corresponding normal tissues. Comparison of L1-CAM expression signals between tumor and normal tissue for (A) ovary and (B) skin. Gene expression data were derived from the GEO database (skin [GSE3189], ovary [GSE6008]). n, Number of biological samples within each group.*

imaging agents in L1-CAM-positive xenografts. [131]Iodine-labeled chCE7 was successfully evaluated for tumor imaging in patients with recurrent neuroblastoma (75).

L1-CAM Antibodies for Treatment of Cancer

As outlined in the preceding sections, L1-CAM is expressed in many types of tumors. In normal tissues, L1-CAM was detected in peripheral nerve bundles and in the collecting tubules of the kidney (31). Therefore the assessment of the toxicology profile of therapy-related L1-CAM antibodies in cross-reacting species is an important issue. Albeit functional domains of the extracellular domain of L1-CAM have been mapped, a systematic study for evaluation of *in vitro* and *in vivo* properties of L1-CAM antibodies directed against different epitopes is still pending. It is reasonable to predict that defined properties of L1-CAM antibodies will be correlated with defined epitopes. Some of the described antibodies were shown to interfere or modulate ERK signaling and thus impact on invasion and proliferation. Inhibition of proliferation of selected tumor cell lines by L1-CAM monoclonal antibodies in the absence of immune effector cells has been shown. The molecular details of these findings, correlation with L1-CAM density on the cell lines, dependency of efficacy on internalization of antigen-antibody complexes and extension to a broader panel of tumor cell lines should be explored in more detail. In addition, transcriptional profiling and proteomics-based analysis of L1-CAM monoclonal antibodies in responding and non-responding tumor cell lines would help to resolve issues such as cell type- and context-dependent *in vitro* and *in vivo* efficacy. *In vivo* efficacy studies have focused on inhibition of invasion and metastasis by L1-CAM monoclonal antibodies (68). The impact of such antibodies on the growth of established xenografts derived from a broad panel of tumor cell lines should be investigated in more detail to come up with a clearer picture of their role in the context of tumor growth inhibition.

The function of the two L1-CAM isoforms in cancer biology, such as their impact on proliferation, differential interaction with ligands, and involvement in migration and invasion, should be explored in more detail in order to design antibodies directed against L1-CAM with optimized efficacy. The generation of monoclonal antibodies selective for each of the two isoforms would allow the IHC profiling of primary tumors and metastatic lesions with respect to the expression of the L1-CAM isoforms. In this context, analysis of matching primary tumors and corresponding metastases might impact on treatment strategies. In addition to therapy of defined tumor entities with L1-CAM monoclonal antibodies, different treatment strategies such as conjugates between the antibodies and cytotoxics, vaccination against non-neuronal epitopes of L1-CAM and treatment of patients with educated T-cells directed against L1-CAM-expressing tumor cells (76) might be explored further.

Mutation of L1-CAM in the cytoplasmic domain (T1274A, S1248A) abrogates ERK activation and blocks cell migration on extracellular matrix proteins (37, 70). These mutations did not augment tumor growth in NOD/SCID mice. Induction of ERK-dependent genes such as β3 integrin, cathepsin-B and several transcription factors and the invasive phenotype were abrogated. Due to the restricted expression of L1-CAM outside the nervous system, monoclonal antibodies and antibody fragments armed with radio-isotopes were evaluated in preclinical radioimmunotherapy approaches (71, 72). For [177]Lu-DOTA-labeled aglycosylated L1-CAM antibody chCE7, the influence of the number of chelators on *in vitro* and *in vivo* properties such as elimination in the blood and uptake in the liver were investigated (73).The highest specific activity was obtained with a chelator-to-antibody ratio of 12. [67]Copper conjugated with chCE7 was evaluated with respect to therapeutic efficacy in orthotopically implanted SKOV-3ip in nude mice. Two mutations were introduced to achieve more rapid blood clearance. Tumor growth inhibition and increase in survival was shown. A combination of unlabeled antibody L1-11A with a subtherapeutic dose of [67]Cu radioimmunotherapy also prolonged survival significantly (74). [177]Lu and [67/64]Cu-labeled F(ab')2 fragments of chCE7 were evaluated as

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