Multiple Splice Variants of Lactate Dehydrogenase C Selectively Expressed in Human Cancer[1, 2]

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ABSTRACT

We applied a combined data mining and experimental validation approach for the discovery of germ cell-specific genes aberrantly expressed in cancer. Six of 21 genes with confirmed germ cell specificity were detected in tumors, indicating that ectopic activation of testis-specific genes in cancer is a frequent phenomenon. Most surprisingly one of the genes represented lactate dehydrogenase C (LDHC), the germ cell-specific member of the lactate dehydrogenase family. LDHC escapes from transcriptional repression, resulting in significant expression levels in virtually all tumor types tested. Moreover, we discovered aberrant splicing of LDHC restricted to cancer cells, resulting in four novel tumor-specific variants displaying structural alterations of the catalytic domain. Expression of LDHC in tumors is neither mediated by gene promotor demethylation, as previously described for other germ cell-specific genes activated in cancer, nor induced by hypoxia as demonstrated for enzymes of the glycolytic pathway. LDHC represents the first lactate dehydrogenase isoform with restriction to tumor cells. In contrast to other LDH isoenzymes, LDHC has a preference for lactate as a substrate. Thus LDHC activation in cancer may provide a metabolic rescue pathway in tumor cells by exploiting lactate for ATP delivery.

INTRODUCTION

The search for tumor-associated antigens as targets for specific immunotherapy of cancer is one of the cardinal quests in tumor immunology. The development of powerful cloning techniques has resulted in the identification of a multitude of novel cancer-associated immunogenic gene products (1–3). Among these, the so-called CT[4] antigens have gained high attention because of their selective expression pattern. The members of this class are not expressed in normal tissues, except for testis, but are present at various frequencies in a broad spectrum of human tumors (4, 5). The first CT antigens were identified by eukaryotic expression cloning using specific T-lymphocyte clones as probes (6, 7). Subsequently, additional members were identified serologically by screening cDNA expression libraries established from tumor specimens with serum of the autologous patients (8–10). As monoclonal antibodies against CT antigens became available, germ cells were identified as the expressing cell population within testicular tissue (11). Several CT antigens are considered valuable candidates for immunotherapy and are currently being assessed as tumor vaccines in clinical trials (12, 13). However, because individual CT genes are expressed in only 20–40% of most tumor types, a polyvalent CT vaccine strategy would be an effective way to increase the number of cancer patients eligible for vaccination and additional tumor antigens need to be defined. To identify tumor antigens beyond those isolated on the basis of their immunogenicity, representational difference analysis was successfully adopted (14–16). As reported here, we used a combined strategy based on data mining and subsequent experimental validation to identify additional germ cell-specific genes activated in cancer. The NCBI GenBank[5] was mined by keyword search for transcripts annotated to be testis-specific full-length transcripts resulting in 143 candidate genes. PubMed was accessed for additional confinement, disclosing published data proving expression in nontesticular normal organs for several of these genes. The remaining genes were subjected to additional analysis combining electronic Northern with RT-PCR in a panel of normal and tumor tissues (Fig. 1). Using this approach, we report here the identification of six novel CT genes. Among them is the testis-specific lactate dehydrogenase isoenzyme LDHC. Most interestingly, this gene is not only activated ectopically in tumor cells. In contrast to other members of the CT gene class, it furthermore gives rise to multiple tumor-specific splice variants expressed exclusively in cancer cells.

MATERIALS AND METHODS

Tissues and Cell Lines. This study was approved by the local ethical review board (Ethikkommission der Ärztekammer des Landes Rheinland-Pfalz). Tissues were obtained during routine diagnostic or therapeutic procedures and were stored at $-80°C$ until use. For studies of transcriptional regulation, cell lines were treated with 2 μM DAC (Sigma) for 72 h or with 100 μM of the siderophore DFO (Novartis) for 24 h.

Data Mining for Selection of Candidate Genes. For identification of candidate genes for expression analysis, a keyword search was performed in GenBank (ENTREZ Search and Retrieval System, Release 08/20/2000) at NCBI for genes annotated to be expressed specifically in testis. A nucleotide sequence file was generated searching for testis-specific gene, sperm-specific gene, and spermatogonia-specific gene, respectively, limited to *Homo sapiens* in the organism field and mRNA for molecule in the GenBank flat-file, excluding all ESTs, genomic sequences, and partial coding sequences. Redundancies were excluded by using sequence homology searching program BLASTN[6] with the homology stringency set high and GeneCard database.[7] Because the search criterion relies on the uncurated annotation provided by the submitting authors, an additional filter is required. Therefore, PubMed was accessed for additional confinement, disclosing published data proving expression in nontesticular normal organs for several of these genes.

eNorthern. All remaining genes were tested for their tissue distribution by eNorthern. Briefly, BLAST search of DNA sequences of interest against EST database at NCBI was performed.[8] The source libraries of ESTs homologous to GOI allow making inferences on the respective gene's tissue distribution. Thereby, genes highly homologous to EST from testis- or tumor-derived
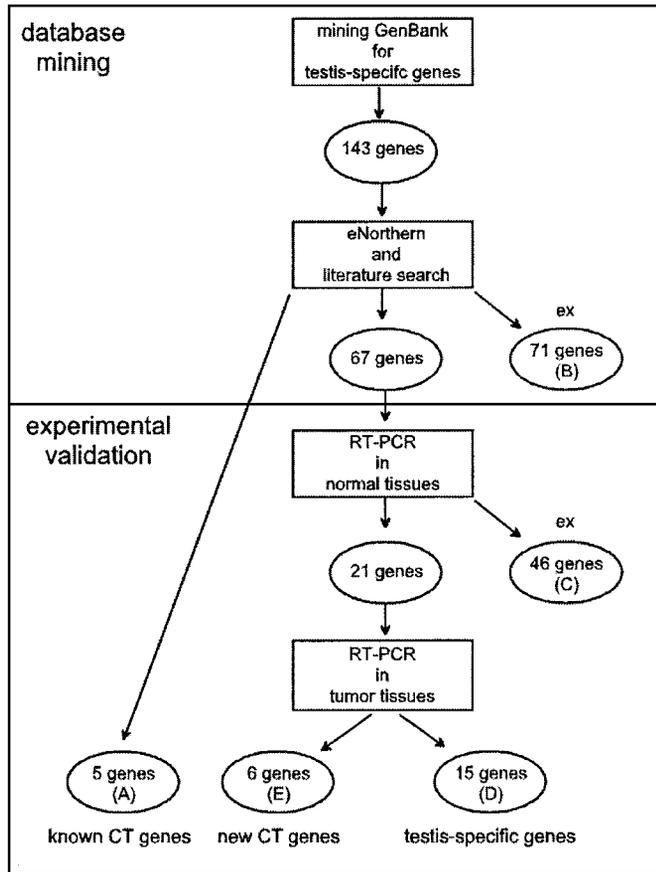
Fig. 1. Data mining for CT-type genes based on keyword search combined with eNorthern and a sequential RT-PCR profiling strategy. Numbers of genes falling into indicated categories are provided. Letters in *parentheses* refer to the columns in supplemental Table 2, which categorizes the gene products.

libraries, but not to EST from nontesticular normal tissues, except placenta and fetal tissues, were identified.

RNA Isolation and RT-PCR. Total cellular RNA was extracted from frozen tissue specimens using guanidine isothiocyanate. Four μg of this RNA was primed with a dT_{18} oligonucleotide and reverse-transcribed with Superscript II (Invitrogen) according to the manufacturer's instructions. Integrity of the obtained cDNA was tested by amplification of p53 transcripts in a 30 cycle PCR (sense, 5′-CGTGAGCGCTTCGAGATGTTCCG-3′; antisense, 5′-CCTAACCAGCTGCCCAACTGTAG-3′; annealing temperature 67°C).

For all GOI, individual PCR assays were designed for specific amplification from first-strand cDNA stocks and are listed in supporting Table 1.

To exclude false-positive PCR products because of contaminating genomic DNA in the RNA preparation, the individual gene-specific primer sets were designed to span exon/intron boundaries. In addition, all primer sets were evaluated by PCR reactions using either genomic DNA or not reverse-transcribed RNA as negative controls.

For PCR analysis of individual gene transcripts, 0.5 μl of first-strand cDNA were amplified with transcript-specific oligonucleotides (Invitrogen) using 1 unit of HotStarTaq DNA Polymerase (Qiagen) in a 30-μl reaction (after 15-min activation at 95°C, 35 cycles of PCR with 1 min at 94°C, 1 min at the respective annealing temperature, 2 min at 72°C, and a final elongation step at 72°C for 6 min). In each experiment, a template-free negative control and testis as positive control were included.

Quantitation by Real-Time PCR. Real-time quantitative analysis for LDHC expression was performed using the ABI Prism 7700 Sequence Detection System instrument and software (Applied Biosystems; Ref. 17). Intron-spanning primers (sense, 5′-GGTGTCACTTCTGTGCCTTCCT-3′; antisense, 5′-CGGCACCAGTTCCAACAATAG-3′) and TaqMan probe (probe, 5′-CAAAGGTTCTCCAAATGT-3′) were designed with Primer Express software (Applied Biosystems). The PCR assay was designed to coamplify all

splice-variants identified with a primer/probe set spanning the junction between exons 1 and 2.

cDNA was generated from 10 μg of total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions. PCR was performed in duplicate with TaqMan Universal PCR Master Mix (Applied Biosystems) using 5 μl of diluted cDNA and gene-specific forward and reverse primers (300 nM) and probe (250 nM; 35 cycles of PCR with 15 s denaturation at 95°C and 1 min at 60°C for annealing/extension). Expression of 18S rRNA in these cDNAs was assessed using the TaqMan RNase Control Reagents (Applied Biosystems) consisting of 18S rRNA specific primers and TaqMan probe. The relative expression level of LDHC transcripts was computed with respect to the internal standard, 18S rRNA, to normalize for variances in the quality of RNA and the amount of input cDNA. The quantity of LDHC transcripts in tumor samples relative to normal tissues was assessed using ΔΔCT calculation (Applied Biosystems).

Analysis of LDHC Transcripts. Amplification was performed from first-strand cDNA derived from a melanoma specimen (Fig. 2*B*, lane 25) scoring as LDHC positive upon typing with primers listed in supporting Table 1. Primers (sense, 5′-TAGCGCCTCAACTGTCGTTGG-3′) and (antisense, 5′-CAACATCTGAGACACCATTCC-3′) used for amplification cover nearly the entire ORF. Amplification products were cloned in pCR2.1 after excision and purification from gel and sequenced. Transcript sequences obtained were aligned to the mRNA sequence of LDHC (NM_002301/NM_017448) and compared with the genomic clones for dissection of the pattern of exon assembly. ORF finder at the NCBI[9] was used for determination of ORFs in splice variants and CLUSTALW software for alignments.

RESULTS

Identification of Candidate Genes by Data Mining. The initial keyword search resulted in a list of 143 independent full-length genes. Among them, five known and previously characterized CT antigens were recognized, validating our data mining approach (supporting Table 2, column A). GenBank EST database was used to establish electronic expression profiles (eNorthern) for the remaining GOI. For 71 of these gene products, significant homologies to ESTs derived from nontesticular normal tissue cDNA libraries were found, ruling out their testis specificity and resulting in their exclusion from additional analysis (supporting Table 2, column B). It was taken into consideration that several cDNA libraries in the public domain are not properly annotated (18).

Distribution of Expression Analyzed by RT-PCR. Sixty-seven GOI appeared testis specific by eNorthern in the sense that matching ESTs derived from nontesticular normal tissues were not found. They were subjected to a two-step analysis by RT-PCR. First, a panel of multiple normal tissue RNA samples (spleen, thymus, mammary gland, liver, ovary, prostate, lymph node, uterus, kidney, thyroid, small intestine, colon, adrenal gland, esophagus, lung, skin, activated peripheral blood mononuclear cells, brain, and testis) was screened with primer sets specific for the respective GOI to reassess testis specificity experimentally. Forty-six genes were detected in normal tissues (supporting Table 2, column C). Several of these gene products like SPAG4, SPAG8, and CCT6B were found to be expressed ubiquitously or in multiple different tissues. Other genes were selectively expressed in single tissues, *e.g.,* TBX22 in testis and thyroid. All these transcripts were excluded from additional analysis because of their expression in normal nontesticular cells.

In a second step, the remaining 21 transcripts without expression in nontesticular normal tissues were additionally analyzed in a panel of cDNAs from different tumor samples (17 lung cancer, 20 breast cancer, 20 colon cancer, 16 melanoma, 8 prostate cancer, 7 renal cell

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[9] Internet address: www.ncbi.nlm.nih.gov/gorf/gorf.html.

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Table 1 *Frequencies of expression of identified CT genes in human tumors*

Tissues	Tested	LDHC 11p15	TPX-1 6p21	SGY-1 19q13	ADAM2 8p11	SPO11 20q13	MORC 3q13
Melanoma	16	7 (44%)	1 (6%)	4 (25%)	1 (6%)	1 (6%)	3 (18%)
Breast cancer	20	7 (35%)	3 (15%)	4 (20%)	0	0	0
Colon cancer	20	3 (15%)	0	0	0	0	0
Prostate cancer	8	3	3	1	2	0	0
Lung cancer	17	8 (47%)	2 (12%)	2 (12%)	1 (6%)	0	3 (18%)
Renal cancer	7	4	1	0	0	0	0
Ovarian cancer	7	3	1	4	0	0	1
Thyroid cancer	4	1	0	0	0	0	0
Cervical cancer	6	5	1	2	0	2	0
Melanoma cell lines	8	5	2	2	0	0	1
Lung cancer cell lines	6	2	1	2	0	2	1

carcinoma, 7 ovarian cancer, 6 cervical cancer, and 4 thyroid cancer) and 14 cancer cell lines (8 melanoma and 6 lung cancer).

Fifteen GOI were not detected in the panel of tumors and therefore classified as strictly testis specific (supporting Table 2, column D), whereas 6 GOI qualified as novel CT genes in that they were detected in various frequencies in different tumor entities (supporting Table 2, column E). All these GOI are not detectable in the normal nontesticular tissues tested with the exception of TPX-1, showing a very faint and inconsistent signal in endometrium (Fig. 2*A*). In tumors expression levels seemed to be heterogeneous (Fig. 2*B*).

Characterization of Novel CT Genes. The group of novel CT genes includes LDHC, a testis-specific lactate dehydrogenase isoenzyme (19), TPX-1, an integral protein of the outer dense fibers and the acrosome of spermatids (20), ADAM2, also known as the metalloproteinase member Fertilin β (21), MORC, the human orthologue of the microrchidia gene (22), SGY-1 (soggy-1), a secreted protein related to the Dickkopf protein family suppressing Wnt-mediated effects during early embryonic development (23), and SPO11, a meiosis-specific protein binding covalently to DNA double-strand breaks (Ref. 24; Table 1, Fig. 2, *A* and *B*). From these, LDHC was detected in virtually all of the tumor types tested, with highest expression frequencies in lung cancer (8 of 17, 47%), melanoma (7 of 16, 44%), and renal cell cancer (4 of 7).

TPX-1 was expressed for example in prostate cancer (3 of 8) and breast cancer (3 of 20, 15%) and Soggy-1 in melanoma (4 of 16, 25%), lung cancer (2 of 17, 12%), breast cancer (4 of 20, 20%), as well as others (Table 1). ADAM2, SPO11, and MORC were detected less frequently, being identified in 4, 5 and 9, respectively, of the total of 119 tumor specimen and cell lines investigated per gene. During preparation of the manuscript, we became aware of a paper reporting expression of ADAM2 in some cases of renal cell carcinoma (25).

Detection and Characterization of New Splice Variants of LDHC. The gene with the highest prevalence of aberrant expression in neoplasms investigated was LDHC. We had taken care that primers were specific for LDHC without cross-amplification of the related ubiquitously expressed isoenzymes LDHA and LDHB. These oligonucleotides coamplified LDHC variant 1 (NM_002301) and variant 2 (NM_017448), both reported to be expressed specifically in testis. They differ in their 5′ untranslated region because of an alternative splicing of exon 1 (Fig. 3). Both variants give rise to the same protein of 332 amino acids. The expected size of the PCR amplification product was 824 bp. Unexpectedly, exclusively in tumor samples at least three additional amplification bands of smaller size were regularly observed (Fig. 2), indicative of the existence of additional splice variants to which we refer as splice variants 3, 4, 5, and 6. To obtain the full lengths of these variants, we amplified the entire ORF from tumor-derived first-strand cDNA. Alignment of the respective products with prototype ORF of LDHC variant 1 and variant 2 and the genomic clones from

chromosome 11 showed either pending of exon 3 (variant 3) or omission of both exon 3 and 4 (variant 4), of exons 3, 6 and 7 (variant 5) or of exon 7 only (variant 6; Fig. 3). The cDNA sequences of the new variants were deposited in GenBank with accession numbers AF401094, AF401095, AF401096 and
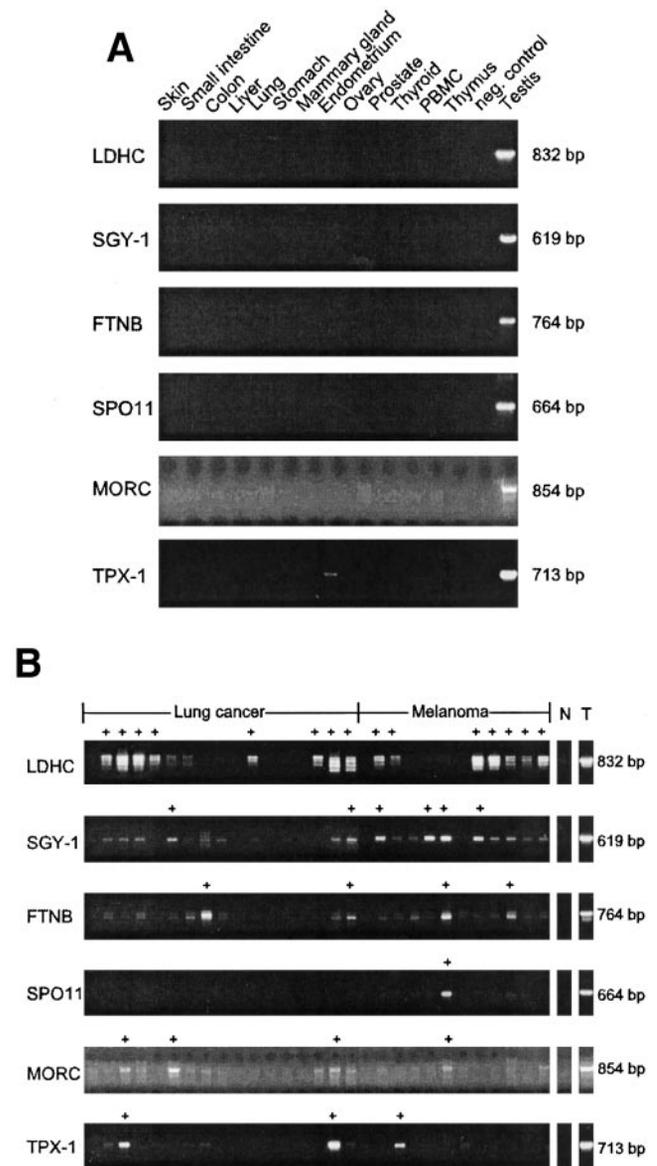

Fig. 2. *A*, RT-PCR analysis of new CT genes in normal tissues. *B*, RT-PCR analysis of new CT genes in tumor tissues and cancer cell lines. *Lanes* considered as positive are marked.
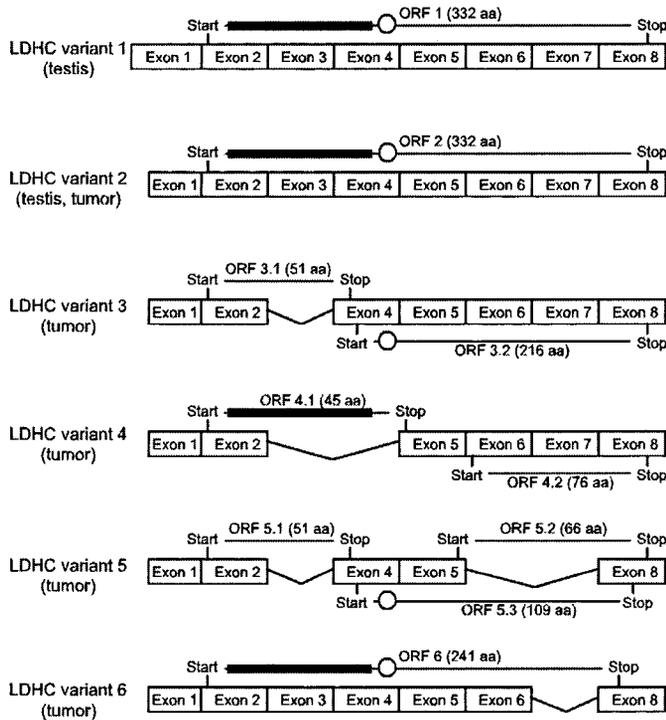
Fig. 3. Schematics of exon usage by the respective LDHC splice variants. The ORFs supposedly translated are tentatively marked. ORF 1 and ORF 2 are identical, so are ORF 1 of variant 3 and ORF 1 of variant 5. *Black bars* represent lactate dehydrogenase NAD binding domain, ○ represents the L-lactate dehydrogenase active site.

AF401097.[10] As pointed out, splice variants 3, 4, 5, and 6 were exclusively detected in cancer but not in testicular tissue. The alternative splicing events in cancer cells affect the reading frames and give rise to seven new putative ORFs (Fig. 3, supporting Fig. 1). In addition to the previously described start site, downstream initiation in exon 4 may occur, resulting in an ORF that is a NH_2-terminally truncated form of the prototype LDHC (ORF 2 of variant 3) or is additionally truncated as well as altered at its COOH terminus (ORF 3 of variant 5). Initiation from a potential start codon in exon 5 (ORF 2 of variant 5) and one in exon 6 (ORF 2 of variant 4) may also result from the depicted splicing events, leading to short proteins missing large parts of the NH_2 terminus.

Noteworthy, according to the criteria established by Kozak (26), the latter potential start codons are in a very strong context for serving as translational initiators.

Of these new putative polypeptides, ORF 2 of variant 3, ORF 3 of variant 5, and ORF 6 (variant 6) bear the catalytic L-lactate dehydrogenase domain for NAD-dependent metabolization of lactate to pyruvate, representing the last step of anaerobic glycolysis (Fig. 3, supporting Fig. 1). Additional analysis using the TMpred and PSORT II algorithm predicted that the putative proteins translated from alternative ORFs might differ in their subcellular localization. Future studies have to dissect whether the predicted proteins indeed are translated and do result in altered structural and enzymatic properties.

Quantification of LDHC Transcript Levels. For comparative quantification of expression levels, a real-time quantitative RT-PCR assay was designed to coamplify all LDHC variants. Amplification of LDHC was performed in a duplex PCR together with 18S rRNA as reference. Because all normal tissues tested, except testis and unexpectedly ovary, displayed undetectable levels of LDHC, their modal was used as calibrator.

Real-time PCR results correlated with findings in RT-PCR and confirmed significant expression of LDHC transcript in tumors (Fig. 4).

Transcriptional Regulation of LDHC Expression. To understand the mechanisms underlying transcriptional regulation of LDHC and its deregulation in tumors, we studied the influence of two factors individually and in combination with each other. Cell lines T2, MCF 7, HCT 116, and MelJuso were treated with DAC and/or with DFO. DAC blocks methylation of CpG islands and has been shown to activate genes that are silenced by promotor methylation. DFO is a strong inductor of HIF-1, a key transcription factor initiating transcriptional hypoxia response programs, including enzymes of the glycolytic pathway. Neither of these compounds resulted in induction of LDHC in the cell lines analyzed (Fig. 5 and data not shown).

DISCUSSION

It has not been unequivocally shown whether antigens identified by cloning strategies based on spontaneous immune responses of cancer patients are indeed the best targets for immunotherapy of cancer. It is well conceivable that tumor-associated gene products that appear as immunologically ignored in patients with manifest tumors (*e.g.,* because of ignorance or deletion/anergy of the respective clones at an early stage of disease) might be superior in a therapeutical setting. Therefore, strategies identifying gene products beyond those that are isolated on the basis of their immunogenicity are attractive. On the basis of the notion of ectopical expression of testis-specific genes in tumors, we investigated whether additional CT type genes could be

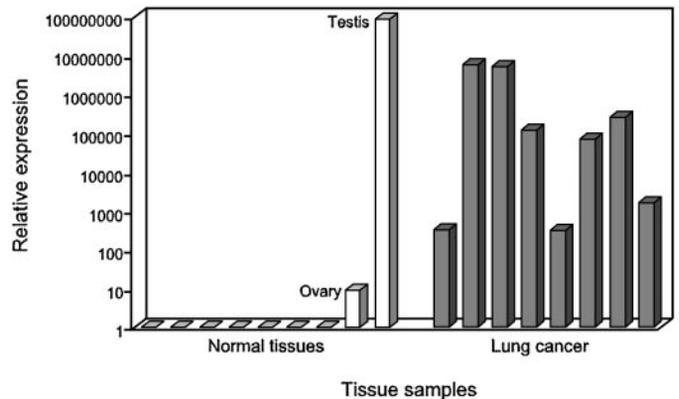Fig. 4. Quantification of LDHC transcript levels in normal tissues and lung cancer by real-time RT-PCR. $\Delta\Delta$Ct calculation was used to calculate transcript levels relative to 18S RNA.
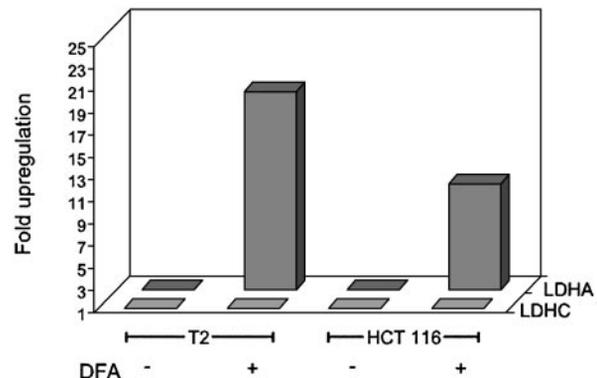

Fig. 5. Real-time RT-PCR analysis of LDHC and LDHA expression in cell lines treated with DFA. Expression is shown relative to respective untreated cells. Induction of LDHA was tested as positive control.

unmasked in the large group of known testis genes simply by assessing their expression in tumor tissue. Surprisingly, the testis-specific expression pattern reported for many genes was not confirmed by RT-PCR in our hands, as we detected expression in nontesticular normal tissues as well. For only 21 of 143 genes discovered by data mining, the prerequisite of strict restriction of expression to testis was confirmed when analyzed in a large panel of normal tissues. Most strikingly, we disclosed by assessing this distinct group of 21 testis genes that ectopical transcriptional activation in cancer is a frequent phenomenon, relating to 6 of them. For CT genes cloned by nonimmunological methods, immunogenicity has not been shown thus far. However, among the novel CT genes unmasked by our approach were three genes, LDHC, ADAM2, (Fertilin β), and TPX-1, originally propagated as potent vaccine candidates for immunocontraception (27), providing an intriguing link of this field to tumor immunotherapy. The immunogenic potential of these proteins has been shown in several immunization approaches to suppress fertility in a variety of mammalian species, including primates. In case of active immunization with LDHC, multiple immunological mechanisms seem to be involved in the strong reversible suppression of fertilization (28). Because serum antibody titers did not correlate with infertility, cell-mediated immunity rather than humoral immunity is thought to be the critical effector mechanism (29). The detection of cancer-restricted splice variants of LDHC is of particular interest. Three of the four tumor-specific splice variants lack at least one of the two domains (NAD binding domain and/or L-lactate dehydrogenase active site) necessary for the catalytic activity of LDHC. It is therefore obvious that a translation of these transcripts would result in truncated and altered molecules without specific enzymatic activity. We do not believe that the polypeptides encoded by these variants (variant 3–5) do have a physiological regulatory function because as they are not expressed in nonmalignant cells. However, it is conceivable that these splice variants are produced erroneously because of an altered transcriptional and/or posttranscriptional regulation in cancer cells (30). The presence of strong Kozak consensus motifs in front of the putative start codons makes it likely that at least some of the identified ORFs are indeed used for translation leading to the generation of tumor-specific neo-polypeptides. Whether the selectively expressed variants of LDHC may give rise to tumor-specific epitopes is currently undergoing investigated.

At least two of the LDHC variants expressed in cancer encode previously described fully active molecules. Our report is therefore the first description of a lactate dehydrogenase activity selectively expressed in tumor but not normal somatic cells. Lactate dehydrogenase catalyzes the interconversion of lactate and pyruvate in the glycolytic pathway. A number of studies have dealt with the tissue specificity and enzymatic properties of the lactate dehydrogenase isoenzymes (31–33), which are encoded by three different genes and have distinct physicochemical, enzymatic, and immunological properties. The LDHA (m-chain) and LDHB (h-chain) gene products combine *in vivo* to give rise to five forms of tetrameric isoenzymes in many somatic tissues (LDH1-LDH5). Despite the widespread use of serum lactate dehydrogenase activity as a prognostic tumor marker (34), the contribution of the individual isoenzymes to this has never been clearly dissected. The homotetrameric LDHC isoenzyme has never been considered as being involved because of its restriction to spermatogenic cells (35).

LDHC is generally more sensitive to pyruvate inhibition and less sensitive to lactate than the other isoenzymes (36). This adaptation seems to be physiologically useful because lactate concentrations in both the male and female reproductive tract fluids are high. Furthermore, spermatids prefer lactate as an energy source over glucose, fructose, and pyruvate (37). The preferred metabolism of lactate by

LDHC provides pyruvate as the starting substrate for the citric acid cycle. Accordingly, spermatids use the citric acid cycle as the major source for energy production (38, 39).

On the other hand, for more than seven decades it is known that a variety of human and animal tumor cells have an increased rate of glycolysis, resulting in the excessive production of lactic acid from glucose. This so-called Warburg effect was a subject of intense investigation, yet its molecular basis is not fully understood.

One of the unanswered questions is as to why some of the energy demand is satisfied by aerobic glycolysis, rather than by mitochondrial oxidative phosphorylation. Besides mitochondrial deficiency in tumors, overexpression of key glycolcytic enzymes has been suggested as underlying mechanism. In this regard, the substrate preferences of LDHC, which is not inhibited even by higher concentrations of lactate, may be beneficial for the metabolism and survival of tumor cells providing a metabolic rescue pathway by using lactate for ATP delivery.

In this study, we also tried to resolve the mechanism of LDHC activation in cancer. It has previously been shown that germ cell-specific genes may escape transcriptional repression in adult somatic tissues in the course of malignant transformation by promotor demethylation (40). However, by experimental treatment of normal and tumor cells with the genome demethylating agent DAC, we could not induce LDHC expression. Recently, a widespread system of oxygen-related gene expression, based on the activation of the transcription factor HIF-1, has been defined (41, 42). Intriguingly, it has been revealed that a short consensus motif in the promotor region of genes for glycolytic enzymes, *e.g.,* the isoenzyme LDHA, could provide the basis for coordinated up-regulation of the pathway upon hypoxic conditions. Although the consensus motif is present in the LDHC promotor region, treatment of cell lines with desferrioxamine, a strong inductor of HIF-1, did not result in expression of LDHC. Activation mechanisms of LDHC might be similarly sophisticated as described for its isoenzyme LDHA for which recent studies unraveled transactivation by c-Myc (43). The availability of LDHC-specific monoclonal antibodies and studies with ectopically expressed LDHC will provide the basis to dissect whether LDHC has a biological significance in the specific metabolic processes observed in tumors.

ACKNOWLEDGMENTS

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