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HOX D13 expression across 79 tumor tissue types

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***HOX* genes control normal development, primary cellular processes and are characterized by a unique genomic network organization. Locus D *HOX* genes play an important role in limb generation and mesenchymal condensation. Dysregulated *HOXD13* expression has been detected in breast cancer, melanoma, cervical cancer and astrocytomas. We have investigated the epidemiology of HOXD13 expression in human tissues and its potential deregulation in the carcinogenesis of specific tumors. HOXD13 homeoprotein expression has been detected using microarray technology comprising more than 4,000 normal and neoplastic tissue samples including 79 different tumor categories. Validation of *HOXD13* expression has been performed, at mRNA level, for selected tumor types. Significant differences are detectable between specific normal tissues and corresponding tumor types with the majority of cancers showing an increase in HOXD13 expression (16.1% normal *vs.* 57.7% cancers). In contrast, pancreas and stomach tumor subtypes display the opposite trend. Interestingly, detection of the HOXD13 homeoprotein in pancreas-tissue microarrays shows that its negative expression has a significant and adverse effect on the prognosis of patients with pancreatic cancer independent of the T or N stage at the time of diagnosis. Our study provides, for the first time, an overview of a HOX protein expression in a large series of normal and neoplastic tissue types, identifies pancreatic cancer as one of the most affected by the HOXD13 hoemoprotein and underlines the way homeoproteins can be associated to human cancerogenesis.**

Key words: *HOXD13*; HOX and multitumor tissue microarray; HOXD13 and pancreas

Class I Homeobox genes (*Hox*, mice; *HOX*, human) are transcription factors mostly involved in embryonic development.[1] They display an unique genomic network organization: 4 compact chromosomal loci (*HOXA* at 7p15.3, *HOXB* at 17q21.3, *HOXC* at 12q13.3 and *HOXD* at 2q31[2]) where 39 sequence corresponding genes can be aligned with each other into 13 antero-posterior paralogous groups. The *HOX* gene network, the most repeat-poor regions in the human genome,[3] is also expressed in normal adult human organs.[4] *HOX* genes appear to regulate phenotype cell identity,[5,6] cell differentiation[7,8] and control primary cellular processes, as proven by the description of congenital,[9] somatic[10] and metabolic[11] diseases involving these genes. Furthermore, besides the already established role of the *HOX* network in hematopoiesis and leukemogenesis,[12] *HOX* genes are implicated in the neoplastic alterations of human solid tissues and organs such as kidney, colon, lung, skin, bladder, liver, breast and prostate.[13,14] New crucial functions have recently been ascribed to *HOX* genes and homeoproteins mostly related to their interaction with miRNAs and ncRNAs to guarantee transcription and export of specific mRNAs.[15,16]

Locus D *HOX* genes are localized on chromosome 2q31-32 and play a role, with its posterior paralogous genes, in limb and digit generation during embryonic development[17] through regulation of mesenchymal condensation. In this genomic area, a global control region (GCR) has been identified upstream of the *HoxD* cluster, harboring cis-regulatory DNA elements able to coordinate the expression not only of *HoxD* genes and their immediate surroundings but also of phylogenetically unrelated genes (*CREB1*, *CREB2*, *Neuro D1*) lying several hundred kilobases from one another.[18]

The most posterior located *HOXD* gene, *HOXD13*, was the first HOX gene to be described as mutated in human synpolidactyly.[19] During normal morphogenesis, *HOXD13* is involved in the determination of the terminal digestive and urogenital tracts.[20–22] *HOXD13* deregulation has been further detected in different tumor types, such as breast cancer, melanoma, cervical cancer and atrocytomas,[23–26] and in the neuro-endocrine differentiation of human advanced prostate cancers.[27]

Here, to immunohistochemically detect the expression of HOXD13 homeoprotein in normal tissues and its potential deregulation along the evolution of specific tumor types, we utilized tissue microarrays (TMAs) including >4,000 different normal tissue and cancer sample types from 79 different tumor categories. *HOXD13* expression has been further validated, at mRNA level, through real-time quantification, for selected tumor types.

We report, for the first time, that an overview of a HOX protein expression in a large series of normal and neoplastic tissues types allows the identification of the specific cancer phenotype related to the HOX protein function. This may represent a general approach useful for the whole *HOX* network to underline the role of the homeoproteins in human cancerogenesis and to allow potential future clinical applications.

Material and methods

Normal tissue array, multitumor tissue array and pancreas tissue array

Three different sets of TMA were utilized for our study: a normal tissue array (NTA) composed of 8 samples of each 76 different normal tissue types ($n = 608$), a multitumor tissue array (MT-TMA) composed of up to 50 samples from 79 different tumor types ($n = 4{,}018$) and a pancreas tissue array (PTA) including 227 ductal carcinomas, 40 samples of pancreatic intraepithelial neoplasia (PanIN3) and 40 specimens of normal pancreatic parenchyma ($n = 307$). Distale bile duct carcinomas, ampullary carcinomas and IPMN-associated carcinomas were not included in the PTA. All ductal carcinomas of the pancreas were classified according to the 6th Edition of the TNM classification (UICC).[28] Selection and characteristics of NTA and TMA have been reported elsewhere.[29]

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TABLE I – COMPARISON OF HOXD13 NUCLEAR HOMEOPROTEIN EXPRESSION IN NORMAL TISSUE AND TUMOR TYPES OF THE SAME ORGAN (MULTITUMOR ARRAY)

Organ	Tissue type	Cases (*n*)	HOXD13 expression		*p*-value[1]	Negative	Positive	*p*-value[2]
			Mean (%)	Median (%)				
Adrenal Gland	Normal	6	0	0	0.014	6 (100.0)	0 (0.0)	0.014
	Cortical adenoma	14	14.3	10		6 (42.9)	8 (57.1)	0.593
	Phaeochromocytoma	23	4.3	0		19 (82.6)	4 (17.4)	0.002
Brain	Normal	12	1.7	0	<0.001	10 (83.3)	2 (16.7)	0.021
	Meningioma	47	4.5	0		35 (74.5)	12 (25.5)	<0.001
	Astrocytoma	45	12.9	0		24 (53.3)	21 (46.7)	0.655
	Glioblastoma multiforme	35	25.1	20		4 (11.4)	31 (88.6)	<0.001
	Oligodendroglioma	21	22.9	10		8 (38.1)	13 (61.9)	0.275
Endometrium	Normal	21	6.5	0	<0.001	14 (66.7)	7 (33.3)	0.127
	Endometrioid adenocarcinoma	46	1.3	10		14 (30.4)	32 (69.6)	0.008
	Serous adenocarcinoma	32	26.3	30		7 (21.9)	25 (78.1)	0.002
Esophagus	Normal	12	3.6	0	0.247	10 (83.3)	2 (16.7)	0.021
	Adenocarcinoma	9	8.9	0		6 (66.7)	3 (33.3)	0.317
	Squamous cell carcinoma	32	10	0		17 (53.1)	15 (46.9)	0.724
	Small-cell carcinoma	1	0	0		1 (100.0)		
Gall bladder	Normal	9	8.9	0	0.178	7 (77.8)	2 (22.2)	0.096
	Adenocarcinoma	44	3.6	0		22 (50.0)	22 (50.0)	1.0
Kidney	Normal	24	9.2	10	0.534	20 (83.3)	4 (16.7)	0.001
	Clear cell renal cell carcinoma	54	4.6	0		37 (68.5)	17 (31.5)	0.007
	Papillary renal cell carcinoma	36	3.3	0		28 (77.8)	8 (22.2)	<0.001
	Chromophobe renal cell carcinoma	10	2	0		9 (90.0)	1 (10.0)	0.011
	Oncocytoma	21	5.2	0		15 (71.4)	6 (28.6)	0.049
Larynx	Squamous cell carcinoma	46	19.3	5		23 (50.0)	23 (50.0)	1.0
Liver	Normal	20	7.5	0	0.887	16 (80.0)	4 (20.0)	0.007
	Hepatocellular carcinoma	85	3.1	0		64 (75.3)	21 (24.7)	<0.001
Lung	Normal	23	2.7	0	<0.001	18 (78.3)	5 (21.7)	0.007
	Squamous cell carcinoma	36	14.4	10		15 (41.7)	21 (58.3)	0.317
	Adenocarcinoma	76	11.8	10		33 (43.4)	43 (56.6)	0.251
	Large cell carcinoma	18	7.2	5		9 (50.0)	5 (50.0)	1.0
	Small cell carcinoma	45	1.1	0		40 (88.9)	5 (11.1)	<0.001
	Bronchioloalveolar adenocarcinoma	7	14.3	20		2 (28.6)	5 (71.4)	0.257
Lymphoid tissue	Normal lymph node	13	1.3	0	0.005	12 (92.3)	1 (7.7)	0.002
	Nodular sclerosis Hodgkin lymphoma	25	9.2	0		17 (68.0)	8 (32.0)	0.072
	Mixed cellularity Hodgkin lymphoma	17	5.3	0		11 (64.7)	6 (35.3)	0.225
	Diffuse large B-cell lymphoma	10	2	0		9 (90.0)	1 (10.0)	0.011
	Non-Hodgkin lymphoma, others	55	1.1	0		52 (94.6)	3 (5.4)	<0.001
Myometrium	Normal	54	7.5	0	<0.001	46 (85.2)	8 (14.8)	<0.001
	Leiomyoma	55	35.5	30		3 (5.5)	52 (94.6)	<0.001
Neuroendocrine tissue	Typical carcinoid of the lung	37	11.6	10	0.868	14 (37.8)	23 (62.2)	0.139
	Extra-adrenal paraganglioma	6	13.3	10		3 (50.0)	3 (50.0)	1.0
Oral cavity	Normal	11	3.6	0	0.359	8 (72.7)	3 (27.3)	0.132
	Squamous cell carcinoma	39	7.7	0		24 (61.5)	15 (38.5)	0.150
Ovary	Normal	4	5.2	2,5	0.863	4 (100.0)	0 (0.0)	0.046
	Serous adenocarcinoma	38	18.7	10		11 (29.0)	27 (71.1)	0.009
	Mucinous adenocarcinoma	18	14.4	10		5 (27.8)	13 (72.2)	0.059
	Endometrioid adenocarcinoma	37	15.7	10		11 (29.7)	26 (70.3)	0.014
Parathyroid	Normal	4	0	0	0.238	4 (100.0)	0 (0.0)	0.046
	Adenoma	39	2.1	0		34 (87.2)	5 (12.8)	<0.001
Peripheral nerves	Neurofibroma	35	5.4	0	<0.001	27 (77.1)	8 (22.9)	0.001
	Schwannoma	46	17.6	10		14 (30.4)	32 (69.6)	0.008
Pleura	Mesothelioma	27	18.1	10		13 (48.2)	14 (51.9)	0.847
Prostate	Normal	22	5.7	0	0.007	16 (72.7)	6 (27.3)	0.033
	Adenocarcinoma, untreated	50	14.2	10		18 (36.0)	32 (64.0)	0.048
	Adenocarcinoma, hormone-refractory	37	8.1	0		19 (51.4)	18 (48.7)	0.869
Skin	Normal	25	45	40	<0.001	23 (92.0)	2 (8.0)	<0.001
	Basal cell carcinoma	65	32.6	30		13 (20.0)	52 (80.0)	<0.001
	Squamous cell carcinoma	37	8.9	10		17 (46.0)	20 (54.0)	0.622
	Malignant melanoma	71	19.4	10		34 (47.9)	37 (52.1)	0.722
	Melanocytic naevus	33	34.2	40		7 (21.1)	26 (78.8)	<0.001
	Fibrous histiocytoma	27	13.7	0		14 (51.9)	13 (48.2)	0.847
	Hemangioma	33	30.6	20		3 (9.1)	30 (90.9)	<0.001
	Kaposi sarcoma	22	45.5	60		0 (0.0)	22 (100.0)	<0.001
	Appendageal tumours (benign)	18	45	40		0 (0.0)	18 (100.0)	<0.001
Small intestine	Normal	15	5	40	<0.001	12 (80.0)	3 (20.0)	0.02
	Adenocarcinoma	15	21.3	20		3 (20.0)	12 (80.0)	0.02
Soft tissue	Normal	8	0	0	0.591	5 (62.5)	3 (37.5)	0.48
	Normal, smooth muscle	12	5	0		7 (58.3)	5 (41.7)	0.564
	Normal, skeletal muscle	157	3.9	0		149 (94.9)	8 (5.1)	<0.001
	Lipoma	28	0.71	0		26 (92.9)	2 (7.1)	<0.001
	Tendon sheath, giant cell tumor	26	38.5	40		2 (7.7)	24 (92.3)	<0.001
	Leiomyosarcoma	4	32.2	40		6 (13.0)	40 (87.0)	<0.001
	Liposarcoma	26	24.2	25		7 (26.9)	19 (73.1)	0.019
	Pleomorphic sarcoma	29	20.3	10		8 (27.6)	21 (72.4)	0.016

TABLE I – COMPARISON OF HOXD13 NUCLEAR HOMEOPROTEIN EXPRESSION IN NORMAL TISSUE AND TUMOR TYPES OF THE SAME ORGAN (MULTITUMOR ARRAY) (CONTINUED)

Organ	Tissue type	Cases (*n*)	HOXD13 expression		*p*-value[1]	Negative	Positive	*p*-value[2]
			Mean (%)	Median (%)				
Testis	Normal	20	7.5	0	0.912	17 (85.0)	3 (15.0)	0.002
	Seminoma	42	6.2	0		23 (54.8)	19 (45.2)	0.537
	Non-seminomatous germ cell tumours	44	7	0		23 (52.3)	21 (47.7)	0.763
Thymus	Normal	8	1.25	0	0.164	7 (87.5)	1 (12.5)	0.034
	Thymoma	37	11.1	0		26 (70.3)	11 (29.7)	0.014
Thyroid	Normal	20	21.9	10	0.052	10 (50.0)	10 (50.0)	1.0
	Follicular adenoma	36	14.4	10		5 (13.9)	31 (86.1)	<0.001
	Follicular carcinoma	51	9.4	10		21 (41.2)	30 (58.8)	0.208
	Papillary carcinoma	31	11.9	10		6 (19.4)	25 (80.7)	<0.001
Urinary bladder	Normal	4	13	10	0.003	3 (75.0)	1 (25.0)	0.317
	Non-invasive urothelial carcinoma	37	26.5	20		5 (13.5)	32 (86.5)	<0.001
	Infiltrating urothelial carcinoma	72	13.8	15		27 (37.5)	45 (62.5)	0.034
Uterus, cervix	Normal	10	3.6	0	0.038	10 (100.0)	0 (0.0)	0.002
	Intraepithelial neoplasia, high-grade (CIN III)	33	13	10		15 (45.5)	18 (54.6)	0.602
Vulva	Squamous cell carcinoma	27	4.4	0		17 (63.0)	10 (37.0)	0.178

The median expression of HOXD13 (10%) was used as a cut-off score to classify cases as negative or positive for HOXD13 protein expression.

[1]*p*-values resulting from comparison of median HOXD13 protein expression levels between tissues of the same organ (Kruskal-Wallis test).–[2]*p*-values resulting from comparison of the number of negative and positive HOXD13 cases for each tissue type (test of equal proportions).

Briefly, tissue samples were derived from paraffin blocks retrieved from the archives of the Institute of Pathology, University Hospital Basel (Switzerland), the Institute of Pathology, University of Bern (Switzerland) and the Department of Biomorphological Sciences, Section of Pathology, University "Federico II" of Naples (Italy). All cases were reviewed by experienced pathologists (L.M.T., L.T., E.K.D.) and representative tissue samples of each tumor category were chosen for TMA construction. Normal tissue samples were derived from resection specimens of non-neoplastic disease and showed a regular architecture of the respective tissue types.

Tissue microarray construction

Tissue samples were fixed in buffered 4% formalin, embedded in paraffin, and used to construct a tissue microarray (TMA). Briefly, hematoxylin-eosin stained sections were made from each selected primary block (donor blocks) to define representative tissue regions. Tissue cylinders (0.6 mm in diameter) were then punched from the region of the donor block with the use of a custom-made precision instrument (Beecher Instruments, Silver Spring, MD). Tissue cylinders were transferred to a 25 mm × 35 mm paraffin block to produce the TMA block used for the study. The resulting TMA block was cut into 3 μm sections that were transferred to glass slides by use of the Paraffin Sectioning Aid System (Instrumedics, Hackensack, NJ). Sections from the TMA block were used for immunohistochemistry.

Immunohistochemistry

Four-micrometer sections of TMA blocks were transferred to an adhesive-coated slide system (Instrumedics, Hackensack, NJ) to facilitate the transfer of TMA sections on the slide and to minimize tissue loss, thereby increasing the number of sections that can be taken from each TMA block. Standard indirect immunoperoxidase procedures were used for immunohistochemistry (ABC-Elite, Vector Laboratories, Burlingame, CA). A rabbit polyclonal antibody was used for HOXD13 detection (1:1,000; ab19866 Abcam, Cambridge, UK). Optimal staining could be achieved after steam cooker pretreatment (5 min, 120°C) in target retrieval solution (DAKO, Glostrup, Denmark; pH 9) for antigen retrieval (3 cycles of 5 min at 650 W). A 3,3′-diaminobenzidine chromogen (liquid DAB; DAKO, Glostrup, Denmark) was used. Nuclei were counterstained with hematoxilin. HOXD13 antibody specificity was assessed through the parallel staining of different MT-TMA slides with paralogous group 13 HOX gene specific antibodies (HOXA13, HOXB13, HOXC13 and HOXD13) (Cantile *et al*., in preparation). The nuclear staining detected specific tumor type expression patterns for each antibody and matched mRNA expression (data not shown). Cytoplasm staining did not fulfill these criteria. Pancreas–TMA slides were immunostained for Neuro D1 (sc-20805, 1:150; Santa Cruz Biotechnology, Santa Cruz, CA), as previously reported.[30]

Evaluation of immunohistochemistry

Immunoreactivity for HOXD13 and Neuro D1 was scored semiquantitatively by evaluating the number of positive nuclei over the total number of cells. Scores were assigned using 5% intervals and ranged from 0% to 100%. The reproducibility of this scoring method between pathologists has previously been demonstrated for TMAs.[31]

RNA extraction and analysis

Total RNA was isolated from frozen biopsies, unrelated to TMA samples, using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. Samples were treated with RNase-free DNase (Qiagen GmbH, Hilden, Germany) to prevent amplification of genomic DNA. A total of 1 μg RNA was subjected to cDNA synthesis for 1 hr at 37°C using the Ready To Go You-Primer First-Strand Beads kit (Amersham Biosciences Europe Gmbh, Freiburg, Germany, cod. 27-9264-01) in a reaction mixture containing 0.5 μg random hexamers (GeneAmp RNA PCR Random Hexamers Set N808-0127 Applied Biosystems, Foster City, CA).

Real-time PCR

TaqMan® analysis was carried out on a 7900HT Sequence Detection System. Singleplex PCR reactions were performed in Fast Gene Quantification in 96-Well Plates with The TaqMan Fast Universal PCR Master Mix (10 μl) in a volume of 20 μl containing 2 μl of cDNA and 1 μl of specific TaqMan Gene Expression Assays for human *HOXD13* (Hs00171253_m1, primers and probe sequences are Applied Biosystems trademarks), according to the manufacturer's directions. All reactions were performed in triplicate. The thermal cycling conditions included a step of 20 sec at 95°C fol-

TABLE II – HOXD13 NUCLEAR HOMEOPROTEIN EXPRESSION IN NORMAL TISSUE AND TUMOR TYPES REPRESENTED IN FIGURES 1 AND 2

Organ	Tissue type	Cases (*n*)	HOXD13 expression		*p*-value[1]	Negative	Positive	*p*-value[2]
			Mean (%)	Median (%)				
Breast	Normal	6	0	0	<0.001	6 (100.0)	0 (0.0)	0.014
	Invasive ductal carcinoma	40	23.5	15		11 (27.5)	29 (72.5)	0.004
	Invasive lobular carcinoma	38	8.9	0		21 (55.3)	17 (44.7)	0.516
	Medullary carcinoma	51	30.4	40		10 (19.6)	41 (80.4)	<0.001
	Tubular carcinoma	20	17	10		9 (45.0)	11 (55.0)	0.655
	Mucinous carcinoma	18	36.1	40		3 (16.7)	15 (83.3)	0.005
Colon	Normal	10	0	0	<0.001	10 (100.0)	0 (0.0)	0.002
	Adenoma, mild dysplasia	21	17.1	20		1 (4.8)	20 (95.2)	<0.001
	Adenoma, moderate dysplasia	55	25.6	20		0 (0.0)	55 (100.0)	<0.001
	Adenoma, severe dysplasia	22	20.7	20		3 (13.6)	19 (86.4)	<0.001
	Adenocarcinoma	38	29.5	40		3 (7.9)	35 (92.1)	<0.001
Salivary gland	Normal	20	2.6	0	<0.001	20 (100.0)	0 (0.0)	<0.001
	Warthin tumour	23	8.3	10		11 (47.8)	12 (52.2)	0.835
	Pleomorphic adenoma	38	37.9	40		1 (2.6)	37 (97.4)	<0.001
	Adenoid cystic carcinoma	32	40.6	0		3 (9.4)	29 (90.6)	<0.001
Pancreas	Normal	16	20.6	40	<0.001	8 (50.0)	8 (50.0)	1.0
	Adenocarcinoma	41	7.1	0		23 (56.1)	18 (43.9)	0.434
Stomach	Normal	12	9.3	10	<0.001	7 (58.8)	5 (41.7)	0.564
	Adenocarcinoma, diffuse type	17	5.9	0		12 (70.6)	5 (29.4)	0.089
	Adenocarcinoma, intestinal type	41	24.6	20		9 (22.0)	32 (78.0)	<0.001

The median expression of HOXD13 (10%) was used as a cut-off score to classify cases as negative or positive for HOXD13 protein expression.

[1]*p*-values resulting from comparison of median HOXD13 protein expression levels between tissues of the same organ (Kruskal-Wallis test).–[2]*p*-values resulting from comparison of the number of negative and positive HOXD13 cases for each tissue type (test of equal proportions).

lowed by a 40 cycles of 95°C for 1 sec and 60°C for 20 sec. All reagents were from Applied Biosystems (Foster City, CA).

The comparative C_t method[32] was employed to determine the human *HOXD13* gene variation, using as reference gene TaqMan Endogenous Controls Human ACTB (β-actin) Endogenous Control (part number 4352935E Applied Biosystems). We identified a calibrator cell line that represents the unitary amount of the target of interest, consequently the samples express *n*-fold mRNA relative to the calibrator. Final amounts of target were determined as follows: target amount $= 2^{-C_t}$, where $C_t = [C_t\,(HOXD13) - C_t\,(\mathrm{ACTB})]_{\mathrm{sample}} - [C_t\,(HOXD13) - C_t\,(\mathrm{ACTB})]_{\mathrm{calibrator}}$.

Statistical analysis

In a first step, *HOXD13* immunohistochemistry expression was evaluated from the multitumor array. For each tissue type and organ, the mean and median expression of *HOXD13*, expressed as a percentage of immunoreactive cells, were noted. The Kruskal-Wallis test was used to identify differences in median expression values within the organ.

In a second step, the median expression value of HOXD13 across all tissue types, namely, 10%, was used to classify tissues as negative or positive for the protein. As the comparison between negativity and positivity was performed for descriptive purposes only, rather than for the analysis of clinical endpoints, the selection of the median value as cut-off score was based on evaluation of the distribution of HOXD13 scores and is commonly used to describe semiquantitative measurements.[33] Differences in the number of negative and positive cases were analyzed using a test of equal proportions.

In a third step, the expression of HOXD13 was evaluated on a pancreas tumor array complete with clinicopathological information and follow-up. Cut-off scores for positivity in this entity were based on receiver operating characteristic (ROC) curve analysis which determines the sensitivity and specificity for a binary endpoint, here survival/nonsurvival, at each possible HOXD13 score thus generating a ROC curve. From the curve, the optimal cut-off score was selected using the (0,1)-criterion to best discriminate between survivors and nonsurvivors and the reliability of the cut-off score was obtained by 200 bootstrapping (resampling) of the data. The cut-off score was determined to be 0% indicating that any degree of immunoreactivity was to be considered a positive case. Survival time differences were analyzed by the Kaplan-Meier method and log-rank test. As the proportional hazards assumption was not met for HOXD13 and Neuro D1, Cox regression analysis could not be performed in multivariable analysis, therefore stratification of cases by pT stage and pN stage was performed to determine the independent prognostic effect of the protein markers. Correlation between NeuroD1 and HOXD13 expression was evaluated by the Spearman Rank correlation. *p*-values <0.05 were considered statistically significant. Box-and-whisker plots of Real Time data were evaluated using *R* statistical software.

Results

HOXD13 expression was evaluated in 3,321 of 4,018 tissue samples (82.7%). The reason for analysis failure is related to the TMA technology, including fractions of missing samples or samples containing only a few tumor cells.

Multitumor array

HOXD13 expression in normal tissue samples. Positive HOXD13 staining was detected in 93 of 578 (16.1%) valuable normal tissue samples (Tables I and II). Only pancreas (8 of 16, 50.0%), thyroid (10 of 20, 50.0%), endometrium (7 of 21, 33.3%) and prostate (6 of 22, 27.3%) demonstrated consistent HOXD13 expression. Adrenal gland, breast, colon, ovary, parathyroid, salivary glands and cervix uteri revealed negative staining in all investigated cases.

HOXD13 expression in tumor samples. Positivity for HOXD13 was detected in 1,584 of 2,743 (57.7%) valuable neoplastic tissue samples. Several tumors including Kaposi sarcoma (22 of 22, 100%), pleomorphic adenoma of the salivary glands (37 of 38, 97.4%), adenoma of the colon (94 of 98, 95.9%), leiomyoma of the myometrium (52 of 55, 94.6%), giant cell tumor of the tendon sheath (24 of 26, 92.3%), adenocarcinoma of the colon (35 of 38, 92.1%), adenoid cystic carcinoma of the salivary glands (29 of 32, 90.6%) and hemangioma of the skin (30 of 33, 90.9%) showed HOXD13 expression in more than 90% of the investigated cases (Tables I and II).

The expression of HOXD13 varied also between distinct tumor types of particular tissue groups. Invasive lobular and tubular

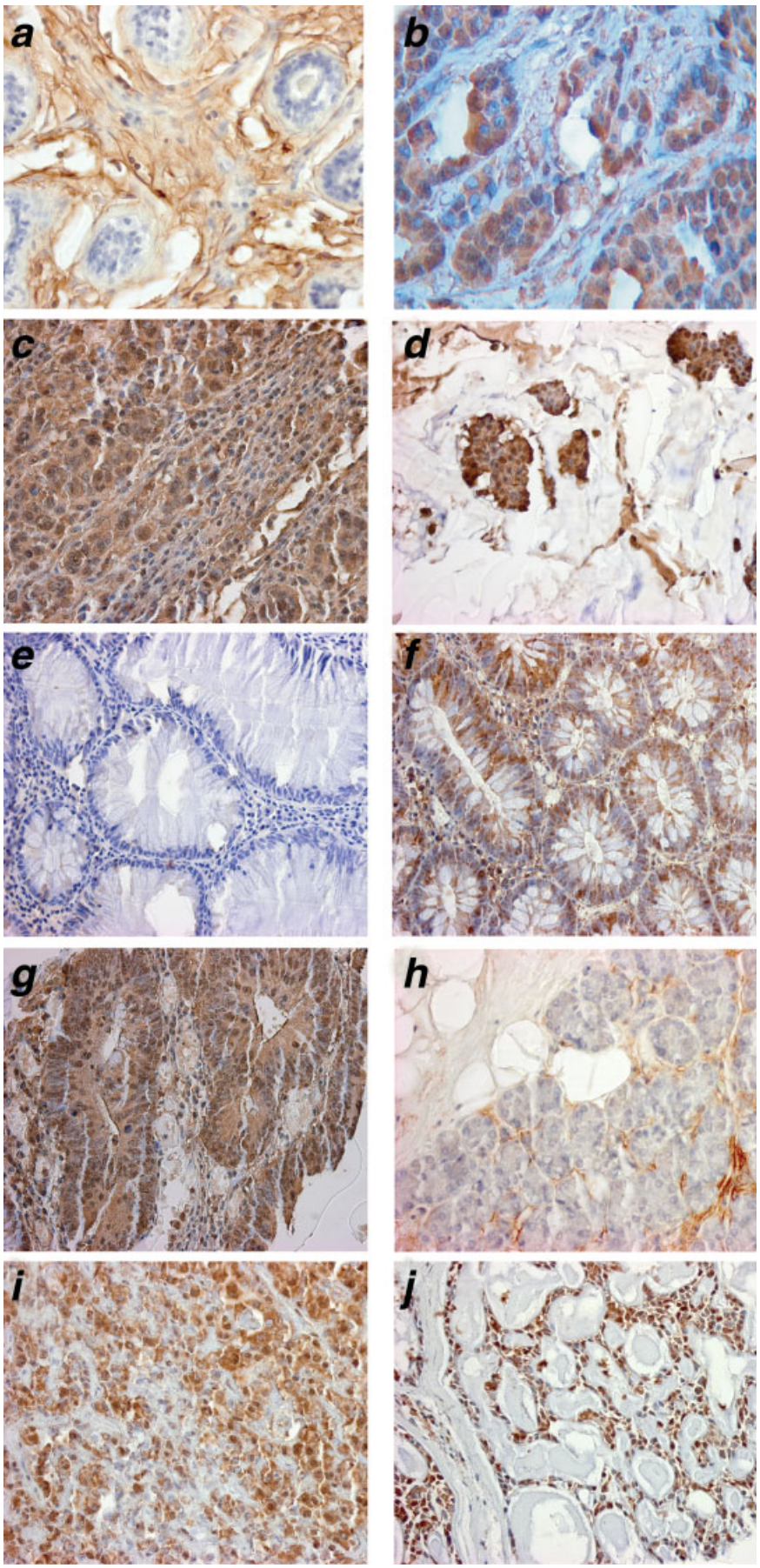


Figure 1 – Nuclear HOXD13 homeoprotein expression in the breast: normal tissue (*a*), invasive ductal carcinoma (*b*), medullary carcinoma (*c*), mucinous carcinoma (*d*), colon: normal tissue (*e*), adenoma (*f*), adenocarcinoma (*g*), salivary glands: normal tissue (*h*), pleomorphic adenoma (*i*), adenoid cystic carcinoma (*j*) (magnification, ×400).

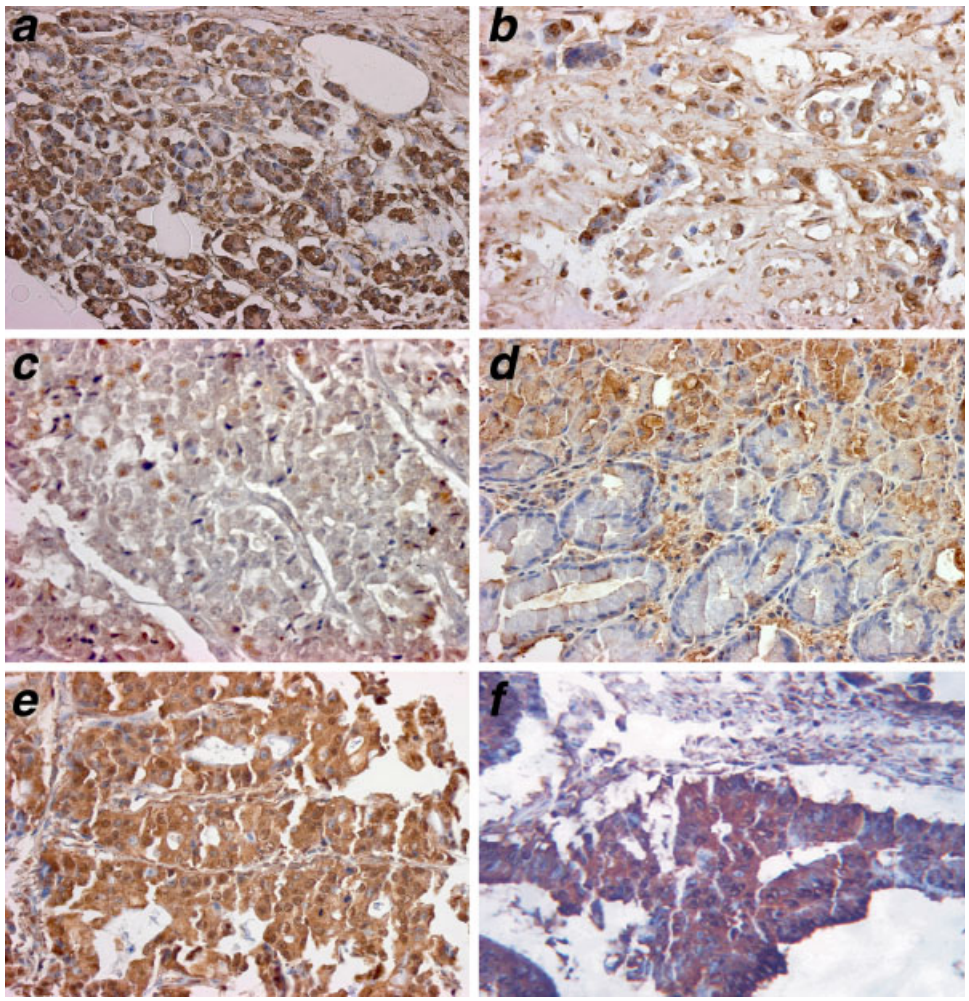


FIGURE 2 – Nuclear HOXD13 homeoprotein expression in the pancreas: normal tissue (*a*), adenocarcinoma (*b,c*), stomach: normal tissue (*d*), intestinal type adenocarcinoma (*e*), diffude type adenocarcinoma (*f*) (magnification, ×400).

carcinomas of the breast showed less frequent HOXD13 positivity than mucinous, medullar and ductal invasive carcinomas (Table II). In brain tumors, HOXD13 stained glioblastoma multiforme and oligodendroglioma strongly (mean expression 25.1% and 22.9%, respectively) and meningiomas (mean expression 4.5%) only weakly (Table I).

Comparison of HOXD13 expression in normal and neoplastic tissue samples. The expression of HOXD13 varied quantitatively (16.1% *vs*. 57.7%) and qualitatively between normal and neoplastic tissue samples of the respective tissue groups. In general, tumor samples stained more consistently for HOXD13 than their normal counterparts. HOXD13 was undetectable in normal colonic mucosa but stained positively 94.8% of adenomas and adenocarcinomas (Fig. 1). Similarly for salivary glands, a significant increase in expression was noted in pleomorphic adenomas and adenoid cystic carcinomas compared to normal submandibular glands (Fig. 1). In skin, only 8% of normal tissues were HOXD13 positive. A considerable increase in positivity was detected in squamous cell carcinomas (54% of cases) and histiocytomas (48.2% of cases), melanoma (52.1% of cases) and appendageal tumors and Kaposi sarcomas (100% of cases; Table I). Thirty two of the 37 cases of noninvasive bladder transitional cell carcinomas (TCC) stained positively for HOXD13 and only with lower frequency in the invasive TCCs and in normal bladder (Table I). In normal prostate, where HOXD13 has been shown to play a role during normal development, a weak staining (6 of 22) was detected. Compared to hormone-refractory prostate cancers, untreated cancers were more frequently HOXD13 immunoreactive with 32 of 50 cases demonstrating positivity (Table I).

In contrast to the general trend, normal pancreas had a significant higher mean HOXD13 expression of 20.6% compared to adenocarcinomas, where only 7.1% of cells stained for the protein (Fig. 2). Similarly, HOXD13 selectively stained stomach intestinal adenocarcinomas (mean 24.6%) *versus* diffuse adenocarcinomas and normal tissue (Fig. 2).

HOXD13 mRNA quantification in normal tissues and tumors

Differences in *HOXD13* expression between normal and tumor tissues were further validated on 48 selected tissue types, by Real-Time PCR quantification (Fig. 3). *HOXD13* mRNA expression corresponded substantially to immunohistochemical protein expression obtained from tissues of the multitumor-TMA. Significant increases in *HOXD13* mRNA expression were observed between normal tissues and various tumor types of the same organ. This was found for brain tumors (glioblastoma multiforme and oligodendroglioma), endometrium (endometroid and serous carcinoma compared to normal tissue), submandibular gland

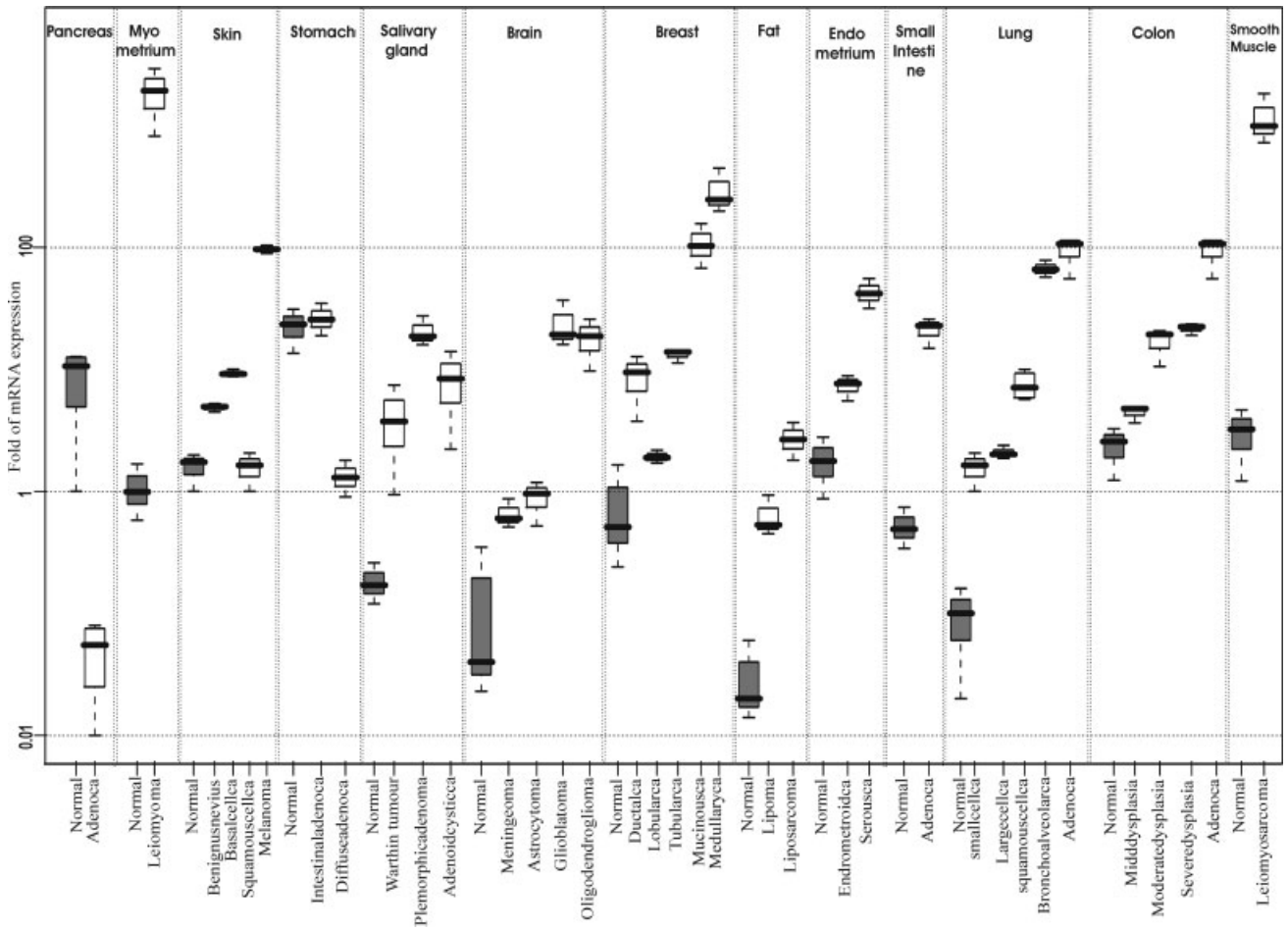


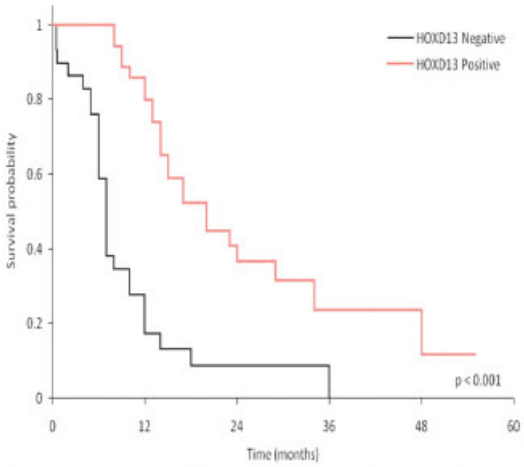
FIGURE 3 – *HOXD13* gene expression by qRT-PCR. Box plots represent the range of *HOXD13* comparative expression values (normal and tumor *vs*. calibrator) analyzed in 13 groups of tissues [48 subgroups ($n \geq 3$)]. Axes represent tumor types *versus* mRNA fold increase. Grey plots are normal tissues.

tumors (pleomorphic adenoma and adenoid cystic carcinoma), breast cancers (medullary and mucinous cancers compared to normal tissue), adenocarcinoma and bronchoalveolar lung carcinoma, melanoma and liposarcoma with respect to normal fat and lipomas. The progressive increase in *HOXD13* mRNA expression was most apparent for tissues of the colon with lowest levels in normal tissues, followed by increased expression in dysplastic tissues and maximum expression in adenocarcinomas. Only in normal pancreas and stomach does *HOXD13* mRNA appear hyper-expressed with respect to pancreas adenocarcinoma and diffuse stomach adenocarcinoma.

Pancreatic tissue array

Association of HOXD13 expression with prognosis. Of the patients with pancreatic ductal carcinoma, whose biopsies are included in the pancreas TMA, 64 had information on survival. The overall 12-month survival rate for these patients in our study was 52.4% (95% CI: 41–63%). Stratifying by HOXD13, patients with negative protein expression ($n = 29$) had a considerable more adverse survival time compared to patients with positive protein expression ($n = 35$). The 12-month survival rates were 17.2% (95% CI: 6–33%) and 79.8% (95% CI: 62–90%) for negative and positive cases, respectively ($p < 0.001$; Fig. 4).

Of the 64 patients with survival information, pT stage was available in 62, of whom 13 had pT1 or pT2 tumors and 49 were diagnosed with pT3 or pT4 disease. Stratifying by pT stage, nega-



No. at risk						
Negative	29	7	1	1	0	0
Positive	35	27	9	3	1	0

FIGURE 4 – Kaplan–Meier survival curve of HOXD13 expression in pancreatic cancer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

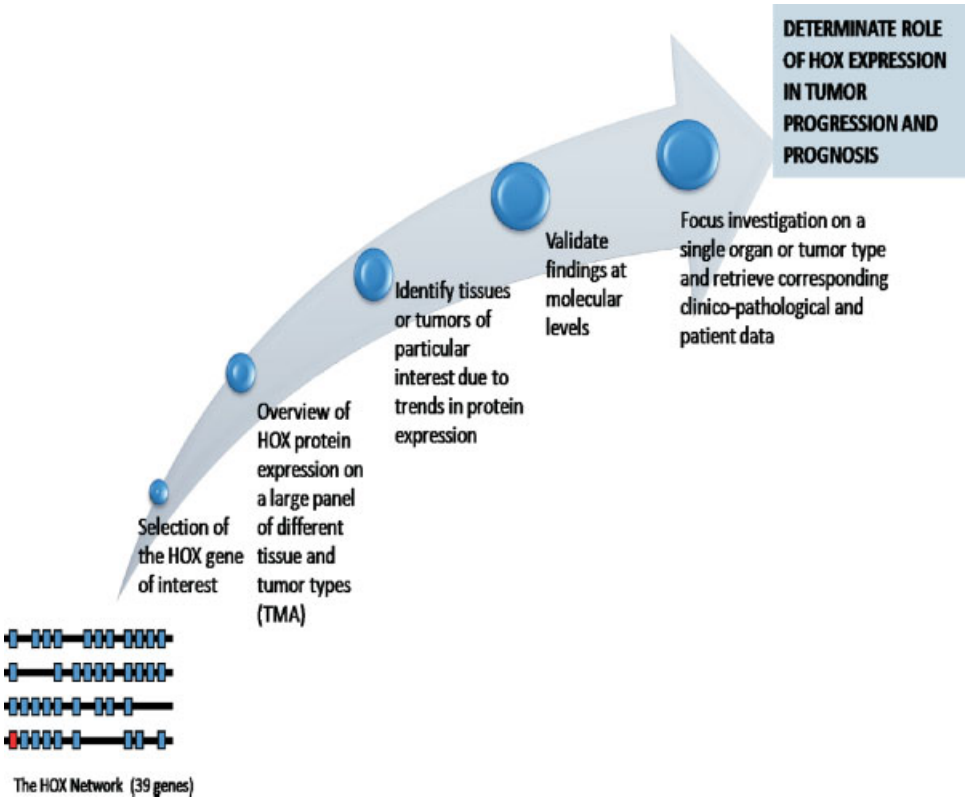


FIGURE 5 – Systematic approach undertaken to study the role of *HOXD13* in human cancers. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tive HOXD13 expression led to significantly worse outcome compared to positive expression of the protein in pT1 or pT2 patients ($p < 0.001$) and also in pT3 or pT4 patients ($p = 0.012$) indicating that the adverse effect of negative HOXD13 expression on prognosis was independent of pT stage.

Information on lymph node metastasis was available in 58 patients of whom 39 had positive lymph nodes (>pN0 disease) and 19 were free of involvement (pN0 disease). In both patients with and without lymph node metastasis, negative expression of HOXD13 led to highly adverse outcome compared to positive HOXD13-expressing cases ($p < 0.001$ and $p = 0.018$, respectively). These results suggest that the effect of negative HOXD13 expression is associated with an adverse outcome regardless of lymph node status.

Differential expression in normal tissues, PanIN and cancer

Differences in the mean HOXD13 expression were evaluated between normal tissue, PanIN and carcinoma. The mean expression was 52% in normal tissue and 19% and 17% in PanIN and carcinoma, respectively ($p < 0.001$) underlining a decreased HOX D13 expression with tumor progression.

Neuro D1 expression

Neuro D1 positivity was linked to more adverse survival time with 12-month survival rates of 59.3% and 63.6% for patients with positive and negative tumors, respectively ($p = 0.044$). This prognostic effect was not found to be independent of T and N stage or of HOXD13 expression. In addition, analysis of HOXD13 and Neuro D1 immunohistochemistry scores were significantly and negatively correlated ($r = -0.21$; $p = 0.009$). The mean Neuro D1 expression in normal tissues, PanIN and carcinomas was 36%, 62% and 59%, respectively, indicating a significant increase in Neuro D1 expression with tumor progression ($p < 0.001$).

Discussion

Posterior located genes in the *HOX* network (paralogous 13 *HOX* genes) generate life-compatible phenotypes, such as synpolydactyly and hand-foot-genital syndrome due to *HOXD13*[19] and *HOXA13* mutations,[9] respectively. Paralogous 13 group *HOX* genes have also been connected to cell proliferation: *HOXA13* with hepatocellular carcinoma evolution, *HOXB13* with breast cancer progression,[34,35] *HOXC13* with hair follicle growth and melanoma progression[36] and *HOXD13* with prostate development and cancerogenesis.[22,27] *HOXA13*, *HOXC13* and *HOXD13* further generate chimerical fusion proteins with Nup 98 involved in human leukemias.[37]

Nuclear HOXD13 homeoprotein expression is detectable in normal stomach, pancreas, small intestine, urinary bladder, kidney, prostate, testis, endometrium, ovary and uterus. These localizations are consistent with the role of *HOXD13* during normal development controlling the morphogenesis of the terminal part of the body including digestive and urogenital tracts.[20] Thus, our data support the involvement of *HOXD13* with gene programs associated with embryonic and adult cell physiology of posterior tissues and organs.

The nuclear HOXD13 expression in 79 tumor types stains specific cancer phenotypes within an organ: meningiomas among brain tumors, medullary and mucinous phenotypes within breast cancers, endometrioid carcinomas among endometrial cancers, lung small cell cancers within lung tumors, stomach intestinal carcinomas among stomach adenocarcinomas, thyroid papillary cancers within thyroid follicular cancers.

Comparison of Multi-Normal and Multi-Tumor-Tissue-Array staining allows the identification of 19 tissues and organs displaying a differential HOXD13 homeoprotein expression. HOXD13 homeoprotein expression matches HOXD13 mRNA expression as detected through real-time PCR on normal tissues and selected cancer phenotypes. The general trend supports an increased

HOXD13 homeoprotein expression in cancer biopsies with respect to normal tissues.

HOXD13 expression is silent in normal breast whereas characterizes medullary and mucinous breast cancer types. It has recently been reported that the expression-ratio of *HOXB13:IL17BR* is a strong, independent predictor of treatment outcome in the setting of adjuvant tamoxifen therapy of breast cancer patients.[38] Paralogous group *HOX* genes are known to complete each other in performing redundant and additive functions. This has already been proven to occur between *HOXD13* and *HOXB13* role in different tumor types.[24,39]

In colon cancer, HOXD13 expression appears to be related to tumor progression. Several observations have documented the role of *Hox* genes in the patterning of the mammalian gastrointestinal tract. Systematic expression profiles have located a *Hox* code with specific *Hox* genes, including *HoxD* genes, in intestine regionalization.[40]

Deregulated HOXD13 expression in several adenocarcinomas on our MT-TMA suggests the potential involvement of this gene with the neoplastic transformation of glandular epithelia. This observation is supported by the described alterations, consequent to *HoxD13* loss of function, during the embryonic development of mutant mice, in the generation of posterior glandular epithelia (ampullary gland, urethral glands, bulbourethral gland, coagulating gland, dorsal and ventral prostate, glandular folding of seminal vesicles[41]).

HOXD13 homeoprotein expression decreases from normal to tumor tissues only in stomach and pancreas. Interestingly, this observation has been confirmed by the detection in pancreas TMA that HOXD13 homeoprotein expression has a significant effect on the prognosis of patients with pancreatic cancer. This effect on outcome is independent of the patients' T or N stage at the time of diagnosis and is in opposition with Neuro D1 expression. HOXD13 is a better prognostic factor than Neuro D1 for pancreatic cancer. We acknowledge, however, that these preliminary findings require validation on a lager cohort of patients and by other groups, due to the sample size of the pancreatic patients in our study and to their relatively short follow-up times.

The chromosomal area 2q31-33 in which the *HOXD* locus is located houses the interactors of cyclic AMP (*CREB1*, *CREB2* and *cAMPGEFII*), specifically active in the developing brain and regulating exocytosis in secretory cells. This same chromosomal area, coordinately regulated by GCR (see introduction), contains the prostate-specific androgen-related gene *PCGEM1*, a miRNA whose expression increases in high-risk prostate cancer patients.[42] In physical contiguity with *PCGEM1* is located the gene *Neuro D1*, whose ectopic expression causes conversion of epithelial cells into neurons[43] and is actively expressed in advanced prostate cancer.[30] *Neuro D1* is further able to interact with the insulin promoter[44] and is between major players of pancreas organogenesis as is expressed in all differentiated and postmitotic pancreatic endocrine cells. It has been postulated that the involvement of *Neuro D1* in promoting cell cycle exit inducing pancreatic progenitor cells to differentiate.[45] Thus, HOXD13 deregulation in brain, prostate and pancreatic cancers appears to be related to the neurogenic and neuroendocrine characteristics of this genomic landscape.

In conclusion, our data identify, through the detection of HOXD13 homeoprotein expression in 79 types of cancer and the validation with parallel mRNA expression, the prognostic role of HOXD13 in pancreatic cancer. Such a systematic approach, as summarized in Figure 5, may represent a useful methodology for future studies aimed at depicting the role of other members of the HOX network in human cancers. Deregulation of HOXD13 supports the important role of *HOX* genes in tumor evolution and represents a starting point for the integrated analysis of specific cancer series, to use *HOX* genes as potential diagnostic, prognostic and predictive markers.

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