Quantitative modeling of clinical, cellular, and extracellular matrix variables suggest prognostic indicators in cancer: a model in neuroblastoma

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BACKGROUND: Risk classification and treatment stratification for cancer patients is restricted by our incomplete picture of the complex and unknown interactions between the patient's organism and tumor tissues (transformed cells supported by tumor stroma). Moreover, all clinical factors and laboratory studies used to indicate treatment effectiveness and outcomes are by their nature a simplification of the biological system of cancer, and cannot yet incorporate all possible prognostic indicators.

METHODS: A multiparametric analysis on 184 tumor cylinders was performed. To highlight the benefit of integrating digitized medical imaging into this field, we present the results of computational studies carried out on quantitative measurements, taken from stromal and cancer cells and various extracellular matrix fibers interpenetrated by glycosaminoglycans, and eight current approaches to risk stratification systems in patients with primary and nonprimary neuroblastoma.

RESULTS: New tumor tissue indicators from both fields, the cellular and the extracellular elements, emerge as reliable prognostic markers for risk stratification and could be used as molecular targets of specific therapies.

CONCLUSION: The key to dealing with personalized therapy lies in the mathematical modeling. The use of bioinformatics in patient-tumor-microenvironment data management allows a predictive model in neuroblastoma.

To clearly distinguish the heterogeneous spectrum of clinical, histological, and molecular markers of cancer, and thereafter determine the markers essential to diagnosing the degree of malignancy and predicting response to therapy, remains a difficult challenge. These essential markers may be elucidated by considering the patient's organism and transformed tissues as holistically interconnected and dependent on microenvironment interactions via biochemical and biophysical signals (1). Mathematical models enable us to integrate measures made at different levels and generate from relatively simple to highly complex computational descriptors

of the disease process of human tumors (2). Digitization of clinical data is necessary to deal with the complex hallmarks of cancer (3). To achieve personalized therapy, wellness and image-defined risk factors must be quantified (4). Hanahan and Weinberg have proposed that eight hallmarks of cancer together constitute an organizing principle that provides a logical framework for understanding the remarkable diversity of neoplastic diseases (5,6). A novel ninth hallmark which includes the aspect of physics has recently been emphasized (7). The identification of these hallmarks by quantifying the structural variations in tumor tissues, at diagnosis as well as during tumor progression and after treatment, using whole histological sections or tissue microarrays with the human eye is a challenging process (8). However, the difficulty can be overcome by forming morphometric data to represent the histological texture and classify the structural changes via sophisticated computational methods (9–11). Cancer cell behavior is altered due to an accumulation of both genetic and epigenetic changes that influence a variety of cellular functions. At the same time, the transformed cells are committed in a complex interaction with the surrounding tumor microenvironment. The tumor microenvironment is comprised of a variety of cell types, lying among a dense network of various extracellular matrix (ECM) fibers merged within the interstitial fluid and gradients of several chemical species, which constantly interplay with malignant cells (12). The stromal cells provide a rich source of growth factors and cytokines, and play a crucial role in modifying the existing connective tissue and depositing new matrix. Tissue scaffolds regulate cell behavior and influence tumor progression (5–7,12,13). Biomathematical analysis of promising biomarker candidates, such as genomic, transcriptomic, proteomic, and epigenomic changes, at the tumor tissue level will play an important role in developing a more powerful mathematical modeling of tumor–microenvironment interactions (14,15). The information obtained can then be linked to tumor progression in patients either refractory to current therapy, or who relapse or who might benefit from novel therapies

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at an earlier stage, in addition to, or instead of, current strategies (16,17). Nevertheless, the mathematical integration of information acquired from clinical and tumor microenvironment interactions into a common description is as difficult to achieve as it is to understand the underlying complexities of

the tumor biology itself, and requires translational researchers, experimentalists and biomathematicians (18,19). Due to the diverse behavior exhibited by neuroblastoma tumors, an algorithm of clinical, histopathological and genetic factors to stratify risk assessment and delineate therapeutic decisions has

Figure 1. Heatmap clustering indicating the statistical relationship between established prognostic markers and the tumor tissue markers analyzed in (a) primary and (b) nonprimary neuroblastomas. Standard hierarchical clustering showing the correlation in two colors: red (negative) and blue (positive). Statistically significant levels are indicated by the gradient between these two colors; brighter colors represent higher significance. Differences were considered statistically significant for P values <0.05 .

Table 1. Summary of statistically significant relationships between factors used for current risk stratification and several of the tumor tissue markers analyzed in primary tumors

			Clinical		Histopathological				Genetics						
			Alive patients	Localized tumors ^a	Disease-free patients	Few neuroblasts	Not anaplastic	GN and iGNB ^a	Absent or scarce SCA ^a	MYCN nonamplified ^a	17q not gained	1p36 not deleted	11q not deleted ^a	Aneuploidy ^a	
ECM	Fibers	Nets	—	—	—	—	—	—	High (P = 0.038)	—	—	—	—	—	
		Aspect reticulin	—	Ovoid (P = 0.049)	—	—	—	—	—	—	—	—	—	—	
		Fractal dimension reticulin	—	Regular (P = 0.038)	—	—	—	—	—	Regular (P = 0.043)	—	—	—	—	
		Perimeter-ratio reticulin	—	Regular (P = 0.038)	—	—	—	—	—	—	Regular (P = 0.038)	—	Regular (P = 0.038)	—	
	Thick bundles	% Collagen	—	—	—	High (P = 0.020)	—	—	—	—	—	—	—	—	
		No./mm ² collagen	—	—	High (P = 0.038)	—	—	—	—	—	—	—	—	—	—
Ground substance		% GAG	—	High (P = 0.048)	—	High (P = 0.019)	—	High (P = 0.013)	High (P = 0.048)	High (P = 0.038)	—	High (P = 0.034)	—	—	
	Tumor	Proliferation	—	—	—	—	Low (P = 0.019)	—	—	—	—	—	—	—	—
		Nuclear size Ki-67 ⁺ (total+ and 2+ plus 3+)	—	—	—	—	—	Small (P = 0.038)	—	—	—	—	—	—	—
Cells		perimeter-ratio Ki-67 ⁺ (total+ and 2+ plus 3+)	—	—	—	—	—	—	—	—	—	—	Irregular (P = 0.019)	—	
	Differentiation	% TH ⁺	—	—	—	—	—	—	—	High (P = 0.016)	—	—	—	—	
		Hypoxia	No. HIF-2α ⁺	—	—	—	—	—	—	—	Low (P = 0.013)	—	—	—	—
Immune	Subgroup lymphocytes	CD45 ⁺ /CD20 ⁺	High (P = 0.045)	—	—	—	—	—	—	—	—	—	—	—	
		No. CD20 ⁺	High (P = 0.038)	—	—	—	—	High (P = 0.038)	—	High (P = 0.038)	—	High (P = 0.038)	—	—	

—: Not statistically significant.

ECM, extracellular matrix; GAG, glycosaminoglycan; GN, ganglioneuroma; HIF-2α, hypoxia-inducible factor-2α; iGNB, intermixed ganglioneuroblastoma.

^aFactors used for current risk stratification.

Figure 2. Mathematical modeling of (a) primary and (b) nonprimary neuroblastomas. Synopsis of patient characteristics with better outcome, considering histopathology, tumor genetics, and tumor tissue prognostic factors. ECM, extracellular matrix; GAGs, glycosaminoglycans; GN, ganglioneuroma; iGNB, intermixed ganglioneuroblastoma; SCA, segmental chromosomal aberrations.

been devised. Prognostic systems are based on a combination of patient age at diagnosis (age cut-off 18 mo); disease stage defined by the International Neuroblastoma Risk Grouping Staging System or the International Neuroblastoma Staging System (20); on histopathological information; *MYCN* copy number and segmental chromosomal aberrations (SCA) such as 1p deletion (D) 11q aberrations or 17q gain (G) (21); and sometimes DNA ploidy. Accordingly, treatment protocols can vary widely (22). The use of precise quantification by imaging technology of pathology specimens to analyze the structural variations emerging from interactions between cells and ECM elements is a sensitive, objective, and reproducible method that can provide important information for inclusion

in computational formulations for risk stratification systems. To illustrate some of these principles, we present the mathematical integration of multimodal data, combining imaging and nonimaging clinical and tumor tissue data, acquired in the context of neuroblastoma studies, suggesting testable hypotheses for making prognostic predictions. Harnessing the synergy of the various forms of data, including clinical variables and biomarker profiles, will allow mathematical predictive models to form part of the holistic approach to medicine in the 21st century.

RESULTS

Combined multiparametric analysis gives us an idea about the relationship between the objectively and subjectively quantified histological features and the clinical and genetics data. In **Figure 1** the bright colors show some tumor tissue indicators from transformed neuroblastic cells and the cellular and the extracellular compartment, emerging statistically as reliable prognostic markers in primary (PT) and nonprimary tumors (N-PT) (**Figure 1a,b** respectively).

These results represent an assessment of prognostic criteria based on classical clinical, histological, and growth patterns (age and stage, amount of Schwannian stroma, neuroblastic cell differentiation, and mitosis-karyorrhexis index) and established genetics (*MYCN* status).

Primary Tumors

A summary of statistically significant relationships between the established prognostic markers (clinical, histopathological, and genetic parameters) and tumor tissue markers (ECM elements and tumor and immune cells) with good prognostic impact in PT is presented in **Table 1**. With respect to established prognostic markers and ECM, a high percentage of reticulin fibers were associated with neuroblastomas with absent or scarce SCA, ovoid reticulin nets were related to localized tumors indicating their crosswise arrangement, and regular reticulin nets were present in localized tumors, tumors without 17q gain (NG) and 11q nondeleted (ND). In addition, tumors with scant neuroblasts, as well as PT from disease-free patients, were surrounded by a large amount of collagen fibers. A high quantity of glycosaminoglycans was also associated with localized tumors, ganglioneuroma (GN) and intermixed ganglioneuroblastoma or tumors with very few neuroblasts, absent or scarce SCAs, *MYCN* nonamplified (MNNA) nor 1p36 deletion (D). Biomathematical analyses also revealed that Ki-67⁺ cells from GN and intermixed ganglioneuroblastoma had small nuclei, whereas tumors without anaplasia presented low expression of Ki-67 and aneuploid tumors showed irregular Ki-67⁺ cell nuclei. In addition, MNNA tumors expressed a high amount of TH and low amount of hypoxia-inducible factor-2α (HIF-2α⁺). Regarding immune cells, a higher quantity of CD20⁺ cells was observed in tumors MNNA, without 1p36 deletion, and PT from alive patients when compared with *MYCN* amplification (A), 1p36 D and PT from deceased patients. **Figure 2a** outlines the characteristics of neuroblastoma patients having a better outcome, considering new and established prognostic markers.

Table 2. Summary of statistically significant relationships between the established hallmarks for current risk stratification and a few of the tumor tissue markers analyzed in nonprimary tumors

				Clinical		Histopathological		Genetics		
				Alive patients	Event-free survival	Few neuroblasts	GN and iGNB ^a	Absent or scarce SCA ^a	17q not gained	11q not deleted ^a
ECM	Fibers	Thick bundles	Angle collagen	Low (<i>P</i> = 0.012)	—	—	—	—	—	—
Cells	Tumor	Proliferation	% Ki-67 3 ⁺	—	—	—	—	Low (<i>P</i> = 0.000)	—	Low (<i>P</i> = 0.027)
			No. mm ² Ki-67 3 ⁺	—	—	—	—	High (<i>P</i> = 0.000)	—	—
			Nuclear size Ki-67 3 ⁺	—	Small (<i>P</i> = 0.003)	—	—	Small (<i>P</i> = 0.000)	Small (<i>P</i> = 0.019)	—
			Aspect Ki-67 3 ⁺	—	Ovoid (<i>P</i> = 0.004)	—	—	—	Ovoid (<i>P</i> = 0.002)	—
			Perimeter-ratio Ki-67 3 ⁺	—	Irregular (<i>P</i> = 0.004)	—	—	Irregular (<i>P</i> = 0.000)	Irregular (<i>P</i> = 0.000)	—
			Roundness Ki-67 3 ⁺	—	Round (<i>P</i> = 0.004)	—	—	Round (<i>P</i> = 0.000)	Round (<i>P</i> = 0.007)	—
			Hypoxia	—	—	—	Low (<i>P</i> = 0.000)	—	—	—
	Immune	Subgroup lymphocytes	CD45 ⁺ /CD3 ⁺	—	—	High (<i>P</i> = 0.026)	—	—	—	—

GN, ganglioneuroma; HIF-2 α , hypoxia-inducible factor-2 α ; iGNB, intermixed ganglioneuroblastoma; SCA, segmental chromosomal aberrations.^aFactors used for current risk stratification.

Nonprimary Tumors

A summary of the statistically significant relationships between the data in N-PT is presented in [Table 2](#). The integration of digitized multimodal data showed that tumors from alive patients presented low crossover of the thick bundles of collagen. In addition, tumors without 11q D and with absent or scarce SCA showed a low amount of Ki-67 positive cells. The Ki-67⁺ cells had small, round and irregular nuclei in tumors with 17q NG, absent or scarce SCA, as well as tumors from patients with long event-free survival. Moreover, GN and intermixed ganglioneuroblastoma had a low amount of HIF-2 α ⁺ cells, whereas tumors with scant neuroblasts presented a high CD45⁺/CD3⁺ cell ratio (*P* = 0.026). [Figure 2b](#) represents the predictive guide for N-PT in neuroblastoma patients with better outcome.

DISCUSSION

We outline the benefit to clinical practice of combining established prognostic clinic-biological markers, and medical imaging technology of tumor tissue elements, in a digitized form, using primary and nonprimary neuroblastoma as an example. The hallmarks of cancer constitute an organizing principle for rationalizing the complexities of neoplastic disease. They include sustaining proliferate signaling, evading growth suppressors, avoiding immune destruction, resisting cell death, enabling replicate immortality, inducing angiogenesis, deregulating cellular energetics, activating invasion and metastasis, and physical interactions of cancer cells with their microenvironments (5–7). To study

the hallmarks, several concepts have been proposed and considered at different scales of complexity, such as protein interactions, biochemical pathways, cellular functions, or whole organism studies (2,18). The mathematical model in which the whole organism is the principal modeling unit is most intricate and implies the intercorrelation of data from patients, tumor and stromal cells, and extracellular tumor matrix. Clinical data used to construct classifiers in cancer can be transformed into mathematical descriptors, based, at least in part, on medical images for inclusion in whole organism modeling (1,23,24). In fact, in neuroblastoma it has become crucial to evaluate the clinical significance of imaging in detecting PT stage and residual disease in quality-controlled prospective clinical trials. The International Neuroblastoma Risk Grouping has developed a staging system based on preoperative features, since diagnostic images provide more robust and reproducible data than those based on operative findings and approaches (20). This new staging system is not intended to be a substitute for the International Neuroblastoma Staging System, and currently, it is recommended that both systems be used in parallel during a transitional period. We utilized the widely used International Neuroblastoma Staging System to define the tumor stage of our retrospective cohort, based on the age of the patient at diagnosis, local and distant extent of the disease, and the resectability of the tumor. Recognition of the widespread applicability of image-defined risk factors (25) as well as other computational clinical data will increasingly affect the development of mathematical models with

Table 3. Summary of cases with relevant clinical, histopathological, and genetic prognostic factors

Clinical data	Histopathological data ^a	Genetic data ^b
PT/N-PT	PT/N-PT	PT/N-PT
Age at diagnosis (months) $\leq 18^c$	Histologic category (INPC)	FISH (INRG) ^d
28.0 $\pm$ 34.3/41.8 $\pm$ 44.4	Neuroblastoma (NB): 70/13	Status MYCN gene ^e :
≤ 18 : 37/4; > 18 : 40/8	Nodular ganglioneuroblastoma (nGNB): 1/0	MNNA: 33/5;
≤ 12 : 29/4; 13–18: 8/0; > 18 : 40/8	Intermixed ganglioneuroblastoma (iGNB): 4/0	G: 8/3;
Stage (INSS) 1; 2; 3; 4: 4s ^c	Ganglioneuroma (GN): 2/1	hetA: 5/1;
1: 18/1; 2: 3/0; 3: 15/5; 4: 35/5; 4s: 6/2	Grade of tumor differentiation ^c	homA: 31/5
1 + 2: 21/1; 3 + 4: 50/10; 4s: 6/2	Undifferentiated (u): 22/1	11q deletion ^c :
Relapse	Poorly differentiated (pd): 44/11	ND: 57/8; D: 15/4
N: 52/6; Y: 26/7	Differentiating (d): 4/1	1p deletion:
Event-free survival time (months)	Not otherwise specified (NOS): 1/1	ND: 32/5; I: 1/1; D: 39/8; I + D: 2/0
40.3 $\pm$ 36.4/54.3 $\pm$ 48.8	Diagnostic	17q gain:
Exitus	uNB: 22/1	NG: 30/2; G: 44/11
N: 48/9; Y: 30/4	pdNB: 44/11	MLPA/aSNP (INRG) ^d
Follow-up time (months)	dNB: 2/0	MYCN amplification
46.2 $\pm$ 36.1/68.3 $\pm$ 44.2	NOS NB: 1/1	N: 31/3; Y: 21/2
	nGNB: 1/0	SCA
	iGNB: 4/0	N: 19/2; Y: 33/3
	Prognostic histologic category (INRG) ^c	Number of SCA
	GN + iGNB: 6/1	1.7 $\pm$ 1.7/1.2 $\pm$ 1.3
	nGNB + dNB: 6/0	Deletion
	pdNB + uNB: 66/12	1p: 26/2; 3p: 4/1; 11q: 12/2
	Tumor cells (%)	Gain
	60.3 $\pm$ 21.5/54.6 $\pm$ 23.8	1q: 3/0; 2p: 16/2; 17q: 29/3
	Anaplasia	Other SCA
	N: 75/14; Y: 2/0	N: 46/5; Y: 4/1
		DNA index ^e D; T; Te; P; O ^c
		1.6 $\pm$ 0.4/1.3 $\pm$ 0.4
		D: 19/3; T: 16/4; Te: 3/2; P: 2/0; O: 7/3
		D + Te: 22/5; T + P: 18/4; O: 7/3

D, diploid; GN, ganglioneuroma; hetA, heterogeneous amplification; homA, homogeneous amplification; I, imbalance; INRG, International Neuroblastoma Risk Group; INPC, International Neuroblastoma Pathology Classification; INSS, International Neuroblastoma Staging System; N, no; N-PT, nonprimary tumors; O, other; P, pentaploid; PT, primary tumors; SCA, segmental chromosomal aberrations; T, triploid; Te, tetraploid; Y, yes.

^aThe histology category was determined according INPC (37) and the INRG (22). ^bGenetic results were interpreted following INRG Biology Committee guidelines (21,34,35); mean $\pm$ standard deviations results from quantitative variables. ^cFactors used for current risk-adapted therapy. ^dFluorescence *in situ* hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA), and single-nucleotide polymorphisms arrays (aSNP) were performed to detect genetic aberrations in tumor cells, as previously described (36,38,39). ^eDNA index was measured using image cytometry.

the whole organism as the modeling unit, and not limited to neuroblastoma alone, but applicable also to other neoplastic diseases.

Technologies for digitizing pathology specimens have advanced significantly in the last decade (8,11,26). Slide scanners are capable of producing high-magnification high-resolution images from whole slides and tissue microarrays within a few minutes. Hence, it is becoming increasingly feasible for basic, clinical and translational research studies to produce thousands of slide images (27). It is fundamental to have a reliable and objective method for precise and quantitative

comparison (28,29). We used fast and accurate methods for digitization and objective quantification. Computer-aided evaluation of pathology image analysis to generate risk stratification have been developed for lymphoma (30), glioblastoma (14), breast and prostate cancers (4). A computer-based grading system to support diagnosis for neuroblastoma that uses grades of differentiation and stromal development was published (29,31). Discrimination of stroma-poor and stroma-rich regions has also been reported using a mathematical model performed on digitized hematoxylin-eosin stained whole-sections with extraction of textural features (32). Genome

Table 4. Tissue markers with possible prognostic impact for novel treatment schemes

Antibody	Function	HC ^d and IHC ^c : dilution (source)	Algorithm
Reticulin fibers ^a	Delicate support net	Gomori ^d	IPP
Collagen fibers ^a	Thick support bundles	Masson's trichrome ^d	IPP
GAGs ^b	Diffusion, barrier–matrix interactions	Alcian blue ^d	PPC
Ki-67 ^a	Nuclear protein for cellular proliferation	1/50 (Dako) ^e	IPP
TH ^b	Enzyme catalyzing the conversion of L-tyrosine to L-DOPA	1/30 (Novus) ^e	PPC
VEGF ^{b,c}	Signal protein stimulating vasculogenesis and angiogenesis	1/100 (Santa Cruz Biotechnology) ^e	PPC
HIF-2 α ^c	Transcription factor responding to changes in available oxygen in ECM	1/1000 (Novus) ^e	—
CD133 ^{b,c}	Cancer stem cell marker	CD133 (Abcam) ^e	NQ
SOX-2 ^{b,c}	Transcription factor: embryonic and neural stem cells	1/50 (Abcam) ^e	NQ
CD3 ^b	Receptor T lymphocytes	1/100 (Dako) ^e	MQ
CD20 ^b	Receptor to enable optimal B-cell immune response	1/100 (Dako) ^e	MQ
CD45 ^b	Receptor playing a crucial role in the function of all leucocytes	1/100 (Dako) ^e	MQ
CD31 ^c	Protein that marks endothelial cell intercellular junctions	1/100 (Dako) ^e	—

—: Not applied.

CD, cluster of differentiation; CD133, CD133 glycoprotein; CD31, platelet endothelial cell adhesion molecule; GAGs, glycosaminoglycans; HC, histochemistry; HIF-2 α , hypoxia-inducible factor-2 α ; IHC, immunohistochemistry; Ki-67, Ki-67 protein; MQ, Membrane Quantification; NQ, Nuclear Quantification; PPC, Positive Pixel Count; TH, tyrosine hydroxylase; VEGF, vascular endothelial growth factor.

^aObjective quantification: ^aImage Pro-Plus (IPP) software. ^bAperio ImageScope software. ^cSubjective quantification. ^dHistochemistry. ^eImmunohistochemistry; Dako Headquarters, Denmark; Novus Biologicals, Littleton, CO; Santa Cruz Biotechnology, Santa Cruz, CA; Abcam, Cambridge, UK.

instability, inflammation, and physical interactions are recently recognized as characteristics crucial to the acquisition of the cancer hallmarks. It is also clear that much biology is executed by virtue of cells of different types interacting with one another or by interactions with environmental signals from tissue scaffolds or other cells generating phenotypic/genotypic changes (5–7,12,13). The linkage of pathology images with genomics presents an opportunity to study morphology in the context of genetics and patient outcome. For this reason, we consider it important to include not only quantification of grade of tumor differentiation, percentage of tumor cells, protein expression of cancer stem cells and differentiated cancer cells, various immune system cell markers, and amount of vascular structures but also quantification of features of the two major components of the ECM (fibrillar proteins and glycosaminoglycan). The predictive model of these characteristics measured by image analysis techniques with current digitization approaches used for risk stratification system has revealed that the clinical, histopathological, and genetic descriptors that define clinically relevant groups are reflected in the essential characteristics of the cellular morphology and ECM architecture. The mechanical properties of ECM play an important role in governing the behavior of neuroblastic, Schwann, and lymphocyte cells. Our results suggest that Shwannian stroma is not the only important factor in the histopathologic analysis of neuroblastic tumors. Multiparametric analysis of other tumor stroma components (reticulin, collagen fibers, glycosaminoglycan, and immune cells) detected by classic histochemistry (HC) and immunohistochemistry (IHC) techniques and incorporated into a quantitative morphological analysis would improve the International Neuroblastoma Pathology

Classification value. The systematic quantification of a larger digitized cohort of neuroblastoma tumors will resolve difficulties in the following critical areas: consistency of results with less intra- and interobserver variability; assessment of intra-tumoral histological and genetic heterogeneity; comparative analysis of biomarker expression and genotypic signatures of tumor cells; evaluation of the tumor cell markers obtained by IHC and genetic techniques; and tumor tissue analysis throughout tumor progression. We present a new approach for whole organism modeling of neuroblastoma patients, combining the computational processing of clinicobiological markers used for prognostic stratification and digitized tissue measurements. Advanced high-resolution and high-content imaging technology and computational studies are being developed to integrate knowledge into medical decision support systems (33). We consider that a contribution to the overall goal in cancer would be to construct high-quality bioinformatics strategies to fuse clinical, histologic, and molecular imaging and nonimaging markers for prognosis, response to therapy, and prediction of recurrence in patients, as well as for identifying patient subgroups for inclusion in future clinical trials for new therapeutic compounds. Routine use of digital medical image and computational technologies will allow extraction of parametric information or new hallmarks that could be used as targets of specific therapies in cancer.

METHODS

Subjects and Prognostic Factors

Ninety-two tumors from 90 patients (78 original and 14 relapse/posttreatment) and 4 control tissues included by duplication in two tissue microarrays were used for the study; producing a total of 184 tumor cores and 8 control cores. This study was approved by the

Figure 3. Flowchart showing the multiresolution neuroblastoma image analysis system. Images belonging to different samples stained with different markers have been quantified by image analysis following a common process including segmentation (differential recognition of the staining) with specific input parameters for each marker and method (**Supplementary Table S1** online) and extraction of some given parameters (output parameters in **Figure 4**). The data obtained have been correlated with clinicobiological prognostic data to obtain potential new parameters and ranges for predictive guide, therapeutic decisions, and/or therapeutic target (**Supplementary Figure S1** online). IPP, Image Pro-Plus.

Experimental Research Ethics Committee of the Spanish Society of Pediatric Hematology and Oncology (File number: 59C18ABR2002; EC number: 2010-021396-81). Participants or their family members/informants signed written informed consent forms. **Table 3** summarizes the cases with respect to the eight markers currently used for risk-adapted therapy (indicated as^c) (21,22,34–39). In addition, we created new variables related to age, stage, histopathology and genetics. In total, 8 clinical, 6 histopathological, and 17 genetic variables were considered. The hypothetical prognostic factors detected by HC and IHC, as well as the function of the markers, staining conditions, and quantitative algorithms used, are shown in **Table 4**.

Image Analysis Measurements

All the slides were digitized at 40× magnification using a ScanScope XT scanner (Aperio Technologies, Vista, CA). The image analysis flowchart is showed in **Figure 3**. Considering the 3 HC and the 10 IHC stains in each tumor core, a total of 2,576 tumor core images were obtained. Two objective methods (1 and 2), and one subjective method (3) were used to quantify the expression of the markers in the digitized images (**Figure 4**). For objective quantification, different algorithms from image analysis software were applied (**Table 4** and **Supplementary Table S1** online). Method 1 used self-designed algorithms with the Image Pro-Plus (IPP) software (MediaCybernetics, Warrendale, PA) for noncellular components, such as reticulin and

Techniques and tissue markers	Variables
	Parameter (definition; unit)/schema
	Quantity
	Percentage of stained area ((sum of all areas × 100) ÷ area core; percentage)
	Number of fibers (number of fibers × 1 mm ² ÷ area core; number/square millimeter)
	Size
	Width (diameter along minor axis of object; micrometers)
	Length (diameter along major axis of object; micrometers)
	Area (area of object; square micrometer)
	Shape
	Aspect (ratio between major axis and minor axis of ellipse equivalent to object)
	Roundness ((perimeter ²) ÷ [4 × π × area])
	Perimeter ratio (ratio of convex perimeter ^c to perimeter)
	Fractal dimension (fractal dimension of the object's outline)
	Branching (number of one-pixel-thick open branches)
	Orientation
	Angle (angle between the major axis of the object and the vertical; degrees)
Method 1	
Objective quantification (D) ^a : IPP software	
Markers: Reticulin fibers Collagen fibers Ki-67 protein ^b	
Method 2	
Objective quantification (D) ^a : PPC software MQ software NQ software	
Markers: Glycosaminoglycans Proteins: TH, CD3, CD20, CD45, VEGF, SOX-2, CD133	
	Quantity
	Percentage of stained area or cells ((sum of positive areas or cells × 100) ÷ area core or total cells; percentage)
	Number of cells (number of cells × 1 mm ² ÷ area core; number/square millimeter)
	Intensity
	Intensity of the staining (0; 1+; 2+; 3+) ^d
	Nuclear: 3 2 1
	Membrane: 3 2 1
	Cytoplasm: 3 2 1
Method 3	
Subjective quantification:	
Markers: Proteins: HIF-2 alpha, VEGF, SOX-2, CD133, CD31	
	Pattern
	Distribution pattern of the positive cells (0: negative; 1: focal; 2: heterogeneous; 3: homogeneous)
	1 2 3
	Quantity
	Range (percentage of stained cells; 0+: <10%; 1+: 10–25%; 2+: 26–75%; 3+: >75%)
	Intensity
	Intensity of the staining (0: negative; 1+: weak; 2+: moderate; 3+: intense)
	Vasculature ^e
	Type (1: microvasculature; 2: micro- and macrovasculature)

^aDigitalization by ScanScope XT scanner at 40x magnification. ^bIn all different intensities of staining, the parameters were measured. ^cPerimeter of the convex outline of the object. ^dIntensity options of the markers. ^eMeasured by CD31 marker.

Figure 4. Description of the parameters measured on the tissue markers related to objective (methods 1 and 2) and subjective (method 3) quantification.

collagen fibers, and for cellular proliferation, such as Ki-67 protein expression. This method measured several parameters to describe the quantity (percentage of stained area; number of objects/mm²), the size (width, length, and area), the shape (aspect or how oval the object is; roundness; perimeter ratio or how regular is the surface of the object; fractal dimension or space-filling capacity of a pattern; branching or how many bifurcations has the object), and the orientation (angle) of stained objects. Method 2 applied previously designed algorithms from the Aperio ImageScope software (Aperio Technologies, Vista, CA): a Positive Pixel Count algorithm for total staining area, Membrane Quantification algorithm for membrane staining, and Nuclear Quantification algorithm for nuclear staining. Parameters measured with method 2 estimated the quantity (percentage of stained areas/cells; number of stained cells), and the staining intensity. Finally, the subjective method (method 3) established the pattern (distribution of positive cells), the range (percentage of stained cells), and the staining intensity, as previously described (40), as well as the presence of vascular structures. A summary of objective and subjective measurements, including mean and

standard deviation, distinguishing PT and N-PT tumors is shown in **Tables 5 and 6** respectively. In total, 23 HC parameters (with objective quantification) plus 28 IHC parameters (15 with objective and 13 subjective quantification) were determined in each tumor core analyzed. Four possible options for staining intensity (indicated in **Figure 3d**) of the IHC markers were considered for each of the 15 objectively quantified parameters. In total, 97 parametric measures from each core were obtained. Given that the 92 analyzed tumors had duplicated cores in tissue microarrays, 17,848 tumor parametric measures were obtained overall.

Statistical Analysis

For a more complete statistical analysis, in addition to the 23 HC and 28 IHC parameters already defined, seven new variables were created. For IHC immune cell markers, the three ratios CD45⁺/CD3⁺, CD45⁺/CD20⁺, and CD20⁺/CD3⁺ were also calculated. With regard to subjectively quantified IHC staining parameters, the sum of staining intensity, range, and pattern was created for each analyzed marker, except CD31. Moreover, in the objectively quantified IHC staining

Table 5. Summary of results from objective quantifications of new tumor tissue markers with possible prognostic impact distinguishing primary and nonprimary tumors

Parameters	Reticulin fibers ^a	Collagen fibers ^a	Glycosaminoglycans ^a	Ki-67 ^b	TH ^b	VEGF ^b	CD133 ^b	SOX-2 ^b	CD3 ^b	CD20 ^b	CD45 ^b
Percentage of positive objects or cells											
PT	4.5±4.4	8.9±8.4	4.6±5.1	5.3±8.4	11.9±18.8	24.8±16.1	44.9±26.9	24.6±16.7	—	—	—
N-PT	7.2±6.1	18.3±17.5	6.8±5.9	6.3±13.2	7.3±12.3	20.4±14.9	40.6±23.6	23.9±4.1	—	—	—
No./mm ²											
PT	643.5±455.8	210.2±129.7	—	448.3±800.5	—	—	—	—	1,136.2±1,718.0	29.3±95.9	3,431.1±5,417.1
N-PT	910.4±633.9	183.9±145.4	—	472.1±1,074.5	—	—	—	—	816.7±1,163.8	12.1±16.8	2,659.6±2,280.5
Width											
PT	7.0±2.0	19.4±6.7	—	5.9±0.7	—	—	—	—	—	—	—
N-PT	7.5±2.9	23.1±6.9	—	5.2±0.8	—	—	—	—	—	—	—
Length											
PT	19.6±4.5	40.5±9.3	—	8.0±0.9	—	—	—	—	—	—	—
N-PT	19.6±4.9	45.9±7.3	—	7.6±1.1	—	—	—	—	—	—	—
Nuclear or object mean area											
PT	60.57±37.05	735.94±2,418.59	—	34.9±7.3	—	—	—	—	—	—	—
N-PT	75.08±62.48	1,963.07±3,714.43	—	31.1±7.9	—	—	—	—	—	—	—
Aspect											
PT	4.3±0.8	3.2±0.7	—	1.4±0.1	—	—	—	—	—	—	—
N-PT	4.3±0.9	3.1±0.4	—	1.4±0.1	—	—	—	—	—	—	—
Roundness											
PT	6.6±1.6	21.1±4.9	—	1.3±0.1	—	—	—	—	—	—	—
N-PT	7.0±1.9	21.4±4.5	—	1.3±0.1	—	—	—	—	—	—	—
Perimeter ratio											
PT	0.91±0.02	0.8±0.1	—	0.96±0.03	—	—	—	—	—	—	—
N-PT	0.91±0.02	0.8±0.02	—	0.96±0.01	—	—	—	—	—	—	—
Fractal dimension											
PT	1.1±0.02	1.3±0.2	—	—	—	—	—	—	—	—	—
N-PT	1.1±0.03	1.3±0.02	—	—	—	—	—	—	—	—	—
Dendrites											
PT	0.1±0.1	3.3±1.1	—	—	—	—	—	—	—	—	—
N-PT	0.1±0.1	2.9±1.0	—	—	—	—	—	—	—	—	—
Angle											
PT	91.5±9.9	87.8±14.3	—	—	—	—	—	—	—	—	—
N-PT	95.3±14.8	90.0±12.6	—	—	—	—	—	—	—	—	—

Mean and SD results from sum of all positive cells/objects in each parameter analyzed.
N-PT, nonprimary tumors; PT, primary tumors; VEGF, vascular endothelial growth factor.
^aHistochemistry. ^bImmunohistochemistry.

Table 6. Summary of results from subjective quantifications of new tumor tissue markers with possible prognostic impact distinguishing primary and nonprimary tumors

Parameters	Number of cases				
	VEGF	HIF-2 α	CD133	SOX-2	CD31
Range (0/1/2/3)					
PT	13/25/22/11	32/18/5/6	18/2/14/39	28/10/17	—
N-PT	5/4/1/2	9/1/0/0	4/1/1/8	5/3/3/3	—
Pattern (n/het/hom/f)					
PT	13/0/53/5	32/16/5/2	18/2/12/41	28/3/25/17	—
N-PT	5/0/6/1	9/1/0/0	4/1/1/8	5/2/4/3	—
Intensity (n/w/m/h)					
PT	13/28/12/18	32/13/2/13	18/3/8/44	28/6/17/22	—
N-PT	5/4/1/2	9/0/0/1	4/1/1/8	5/2/3/4	—
Type of vasculature (mi/b)					
PT	—	—	—	—	62/8
N-PT	—	—	—	—	11/2

(0) <10%, (1) 10–25%, (2) 26–75%, (3) >75%; (n) negative, (f) focal, (w) weak, (m) moderate, (h) high; (mi) micro, (b) both macro/micro.

HIF-2 α , hypoxia-inducible factor-2 α ; N-PT, nonprimary tumors; PT, primary tumors; VEGF, vascular endothelial growth factor.

parameters, in addition to the four intensity categories distinguished, two new categories were created: the sum of all positive cells (1+, 2+, and 3+), and the sum of 2+ and 3+ positive cells. Subsequently, these 58 tissue marker variables (Tables 4–6) plus the 31 variables derived from the patient's clinical history, the histopathology and the tumor genetic information (Table 3) were analyzed. Linear regression models were used to assess the dependence of the clinical variables upon genetic and histopathologic markers. All continuous variables were log transformed where necessary to achieve a more normal or symmetric distribution. A regression model was constructed for each analyzed pair of variables. The Wald statistic was used to derive *P* values accounting for the significance of the slope in the model, being different from zero. Thus, for continuous variables, linear regression models provide the same information as the Pearson correlation. Linear models also have the advantage of being applicable when the independent variable, the marker in our case, is binary or discrete. Thus, we can explore the relationship of our clinical variables with each of our markers, independently of whether the marker is a continuous or a discrete variable. This paradigm is appropriate to our study as half our variables, the genetic ones, are discrete while those derived from imaging are generally continuous. Separate analyses were carried out for PT and N-PT. All *P* values were corrected for multiple testing to control the false discovery rate. The standard procedure described in Benjamini–Hochberg was used for this study (41). All analyses were done using R statistical software (42). A sample classification would be possible after processing a whole set of data for a single patient (Supplementary Figure S1 online)

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/pr>

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