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Original Article

ORIGINAL ARTICLE

NDRG1 deficiency is associated with regional metastasis in oral cancer by inducing epithelial–mesenchymal transition

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Abstract

Regional metastasis is the single most important prognostic factor in oral squamous cell carcinoma (OSCC). Abnormal expression of N-myc downstream-regulated genes (*NDRGs*) has been identified to occur in several tumor types and to predict poor prognosis. In OSCC, the clinical significance of deregulated NDRG expression has not been fully established. In this study, NDRG1 relevance was assessed at gene and protein levels in 100 OSCC patients followed up by at least 10 years. Survival outcome was analyzed using a multivariable analysis. Tumor progression and metastasis was investigated in preclinical model using oral cancer cell lines (HSC3 and SCC25) treated with epidermal growth factor (EGF) and orthotopic mouse model of metastatic murine OSCC (AT84). We identified NDRG1 expression levels to be significantly lower in patients with metastatic tumors compared with patients with local disease only ($P = 0.001$). NDRG1 expression was associated with MMP-2, -9, -10 ($P = 0.022$, $P = 0.002$, $P = 0.042$, respectively) and BCL2 ($P = 0.035$). NDRG1 lower expression was able to predict recurrence and metastasis (log-rank test, $P = 0.001$). In multivariable analysis, the expression of NDRG1 was an independent prognostic factor (Cox regression, $P = 0.013$). In invasive OSCC cells, NDRG1 expression is diminished in response to EGF and this was associated with a potent induction of epithelial–mesenchymal transition phenotype. This result was further confirmed in an orthotopic OSCC mouse model. Together, this data support that NDRG1 downregulation is a potential predictor of metastasis and approaches aimed at NDRG1 signaling rescue can serve as potential therapeutic strategy to prevent oral cancer progression to metastasis.

Introduction

Oral squamous cell carcinoma (OSCC) is an aggressive malignancy characterized by local invasiveness and high propensity to locoregional recurrence (1). The presence of cervical nodal metastasis is the single most important prognostic factor in OSCC and has direct impact in the treatment decision. A significant proportion of oral cancer patients presents with occult

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Abbreviations

EMT	epithelial–mesenchymal transition
IHC	immunohistochemistry
MMPs	members of extracellular proteinases
NDRGs	N-myc downstream-regulated genes
OSCC	oral squamous cell carcinoma
PBS	phosphate-buffered saline
TCGA	The Cancer Genome Atlas

neck metastasis. In these cases, the accurate prediction of their occurrence becomes crucial (2,3).

The metastatic process involves enhanced cellular plasticity and dedifferentiation of epithelial cells to acquire motile invasive properties through oncogenic transformation (4). Numerous studies pointed to the importance of N-myc downstream-regulated genes (NDRG) expression for the cells to maintain their stability and epithelial phenotype (5). NDRG is a family with four intracellular proteins (NDRG1-4), each consists of 340–394 amino acids sharing a common NDR domain with 53–65% identity, but distinct N and C terminal domains (6). All family members are characterized by an α/β hydrolase-fold motif; however, the precise molecular function of these family members has not been fully elucidated. NDRGs have been involved in diverse biological functions with impact on cell survival and proliferation (7), response to hypoxia (8) and metastasis development (9,10).

Emerging evidence suggested *NDRG1* as a metastasis suppressor (11–13). During metastatic process, NDRG1 is suppressed to enable cellular invasive properties, such as activation of members of extracellular proteinases (MMPs) and morphological changes probably through the epithelial–mesenchymal transition (EMT) activation (14,15). NDRG1 is also found to regulate actin cytoskeleton re-organization and subsequent reduction of cancer cell migration (11,14). *NDRG1* may regulate different signaling pathways resulting in interruption of major metastasis-associated functions, including EMT, cytoskeleton remodeling and subsequent migration and invasion (16). Noticeable, rescue of NDRG1 is reported to inhibit EMT phenotype (14,15). Although some of these molecular pathways have been explored, the clinical significance and the role of NDRG1 are only partially elucidated, especially in OSCC where the results are contradictory. In this study, we evaluate the clinical and prognostic importance of NDRG1 in 100 patients with long-term follow-up and we investigated relationships between NRDG1 expression and OSCC cell invasiveness *in vitro* and in orthotopic mouse model. Epidermal growth factor (EGF)-induced downregulation of NDRG1 is enhancing OSCC migration and invasion. Furthermore, NDRG1 downexpression was able to promote metastasis *in vivo* by upregulating the expression of MMPs and activation of EMT.

Materials and methods

Study population

After approval of the Human Research Ethics Committee of A.C. Camargo Cancer Center (Sao Paulo, Brazil) and Jewish General Hospital (McGill University, Montreal, Canada), treatment-naive patients with histologically proven OSCC, without a second primary tumor, were included in the study. According to ethical guidelines, patients provided written informed consent and clinicopathological data and samples were handled in a coded fashion.

One hundred OSCC paraffin-embedded tissue specimens were collected from 28 patients who had recurrence or regional metastasis and 72 OSCC patients with no evidence of progression and good outcome after a follow-up for up to 120 months (Table 1). P16 results were positive in 4% of patients with OSCC. Tumor was staged according to the American Joint Committee on Cancer and *Union Internationale Contre le Cancer* (AJCC/UICC), TNM Staging for head and neck cancer, 7th edition 2010 (17). The medical records of all patients were examined to obtain detailed clinicopathological data [age, gender, race, social habits (smoking and alcohol consumption), local tumor size, lymph nodes involvement, grading, vascular embolization, perineural infiltration, lymphatic permeation and extracapsular spread]. During follow-up, all locoregional recurrences were histologically confirmed. The histological grade was determined on the basis of classification proposed by the World Health Organization (18). Vascular embolization was classified according to the presence or absence of neoplastic cells, located both in the wall and in lumen of the blood or lymphatic vessels, perineural infiltration considered present when the tissue adjacent to the peritumoral and/or intratumoral nerves were involved by the neoplastic cells.

Table 1. Clinicopathological characteristics from patients with oral cancer (OSCC)

		Paraffin-embedded samples, *n* (%)	
Variable	Category	Non-metastatic	Metastatic
Age	<55 years old	39 (54.2)	15 (53.6)
	≥55 years old	33 (45.8)	13 (46.4)
Gender	Male	56 (77.8)	25 (89.3)
	Female	16 (22.2)	3 (10.7)
Smoking habit	No	7 (10.8)	1 (5)
	Yes	58 (89.2)	19 (95)
Alcohol consumption	No	16 (24.6)	2 (10)
	Yes	49 (75.4)	18 (90)
T category	T1 + T2	28 (39.4)	5 (22.7)
	T3 + T4	43 (60.6)	17 (77.3)
N category	N0	45 (63.4)	1 (4.3)
	N+	26 (36.6)	22 (95.7)
Recurrence or metastasis	No	72 (100)	0
	Yes	0	28 (100)
Death	No	43 (59.7)	6 (21.4)
	Yes	29 (40.3)	22 (78.6)

T, tumor size; N, lymph nodes involvement.

Tissue Microarray Platform Platform

Core biopsies were taken from previously defined areas, with a Tissue Microarrayer (Beecher Instruments Inc.). For multiple primaries, the index tumor was used. Tissue cores with a dimension of 1.0 mm were punched from each specimen and arrayed in duplicate on a recipient paraffin block. Each core was spaced 0.2 mm apart. After cutting (3 μm) on the recipient block and transferring with an adhesive tape to coated slides for subsequent UV cross-linkage (Instrumedics Inc.), the slides were dipped in a layer of paraffin to prevent oxidation and kept at –20°C (19).

Immunohistochemistry

The immunohistochemistry (IHC) reaction was done as described previously (19). Sections were deparaffinized and rehydrated in graded ethanol solutions. Thereafter, sections were treated with endogenous peroxidase quenching (0.3% H_2O_2 for 15 min) and blocked for avidin/biotin (DAKO® Biotin-blocking System, Dako Corp.) and protein (DAKO® Protein Block Serum-Free, Dako Corp.), 20 min each prior to primary antibody incubation. Pressure cooker antigen retrieval consisted of one period at 125°C for 30 min and 90°C for 10 min in 10 mM citric acid solution (pH 6.0) followed by a washing step with phosphate-buffered saline (PBS). The incubations with the primary antibodies diluted in PBS were conducted overnight at 4°C for anti-NDRG1 (1:1000; Cell Signaling), anti-MMP-1 (Neomarkers, 1:400), anti-MMP-2 (Neomarkers, 1:200), anti-MMP-9 (Neomarkers, 1:200), anti-MMP-10 (Neomarkers, 1:50), anti-MMP-12 (Abcam, 1:500), anti-MMP-13 (Neomarkers, 1:100), anti-E-cadherin (Dako, 1:400), anti-vimentin (Abcam, 1:100), anti-B-catenin (Thermo Fisher Scientific Inc., Fremont, 1:500) and anti-BLC2

(Dako, 1:100). The sections were washed and incubated with secondary antibodies for 30 min (AdvancedTM HRP Link, Dako Cytomation) followed by the polymer detection system for 30 min at room temperature (AdvancedTM HRP Link, Dako Cytomation). Reactions were developed with a solution containing 0.6 mg/mL of 3,3′-diaminobenzidine tetrahydrochloride (Sigma–Aldrich) and 0.01% H_2O_2 and then counterstained with Mayer's hematoxylin, dehydrated and mounted with a glass coverslip. Positive and negative controls were included in all reactions, as described previously (19). The negative control consisted of (i) omitting the primary antibody and incubating slides with PBS and (ii) replacing the primary antibody with normal mouse serum. The immunohistochemical reactions were performed in duplicate on different tissue microarray platform levels, representing 4-fold redundancy for each case. The second slides were 25 sections deeper than the first, resulting in at least 250 μm of distance between the two sections representing different cell samples for each tumor (19).

Immunohistochemical scoring

Immunohistochemical scoring was blinded to the outcome and clinical aspects of each tumor specimen, as described previously (19). After scanning each tumor specimen in low power field to choose the most stained area, at least five fields were evaluated using high power. The presence of a clearly visible dark brown precipitation was considered an immunoreaction. Evaluation of the molecular markers included the proportion of reactive cells within the tumors and the staining intensity. The samples were classified as negative (no visible reaction or <5% of positive cells) or positive reaction (weak and strongly positive present in ≥5% of stained tumors cells). Protein expression intensity was evaluated semiquantitatively using a four-tiered system (0, negative; 1, weak; 2, moderate; and 3, strong). For statistical analysis, the samples were categorized into two groups: negative or weak (score ≤ 1) and moderate/strongly positive cases (score > 1).

Primers and quantitative real-time RT–PCR

cDNAs were synthesized from 1 μg of total RNA using Superscript II reverse transcriptase and random primers (Invitrogen). Primer set sequences was designed in Primer Express 3.0 software (http://bioinfo.ut.ee/primer3-0.4.0/) as following *NDRG1* (forward, 5′-CTCCTGCAAGAGT TTGATGTCC-3′; reverse, 5′-TCATGCCGATGTCATGGTAGG-3′). Quantitative real-time RT-PCR amplification was conducted in a total volume of 20 μL, using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City) and quality controls as proposed by MIQE Guidelines (20). The reactions were carried out in triplicate. *GAPDH* was the most stable control gene from three endogenous genes tested (*GAPDH* (forward, 5′-AATGAAGGGGTCATTGATGG-3′; reverse, 5′-AAGGTGAAGGTCG GAGTCAA-3′), *ACTB* (forward, 5′-GCACCCAGCACAATGAAG-3′; reverse, 5′-CTTGCTGATCCACATCTGC-3′) and *HPRT1* (forward, 5′-GAACGTCTTGC TCGAGATGTGA-3′; reverse, 5′-TCCAGCAGGTCAGCAAAGAAT-3′), using the geNorm algorithm (20). Fold differences in the relative gene expression were calculated using Pfaffl model (21).

Cell culture and reagents

Oral human cancer cell lines HSC3 and SCC25 were purchased from American Type Culture Collection (ATCC) and the mouse cell line (AT84) was established and characterized by our group (22). All the cell lines were tested once purchased, using the 3730xl DNA Analyser (Applied Biosystems). They were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 1% non-essential amino acids and penicillin/streptomycin. Cells were cultured at 37°C with 5% CO_2. Cell line use was limited to passage nine or lower and periodically authenticated by morphologic inspection and mycoplasma testing. Cells were stimulated with EGF (50 ng/mL, Sigma), a potent inductor of EMT and consequently tumor invasion (19,23,24) for a period of 0 and 2 h.

Transwell migration assay

For the transwell migration and invasion assays, 1×10^5 cells were suspended in serum-free medium containing EGF (50 ng/mL) (Invitrogen) and placed in the upper chamber for 24 h, and in the upper chamber of a 10% Matrigel-coated membrane for 48 h, respectively, whereas the lower chamber was filled with serum-free medium with or without EGF (50 ng/mL, Sigma). For both experiments, cells in the upper chamber were removed, and the migrated cells at the bottom of the membrane were fixed with 4% paraformaldehyde and stained with hematoxylin. In both assays, the number of invading cells was determined in 10 randomly chosen fields under the microscope in three independent experiments.

Western blotting

Total cell extracts were used for western blotting as described earlier (23,24). The primary antibodies used were against human NDRG1 (Cell Signaling); E-cadherin (Dako); vimentin (Abcam); B-catenin (Thermo Fisher Scientific Inc.); N-cadherin (Dako); FAK (Millipore); BLC2 (Dako) and GAPDH (Sigma–Aldrich). All primary antibodies were used at a 1:1000 dilution. Anti-mouse and rabbit secondary antibodies (Sigma–Aldrich) were used at a 1:10 000 dilution.

Tumor metastasis assay in animal model

Animal handling and procedures were conducted in compliance with institutional and federal Canadian guidelines and approved by McGill University. Tumor metastasis was determined by orthotopic injection of AT84 cells into 4- to 6-week-old male Balb/c. Briefly, each animal was injected orthotopically into the tongues with 10^6 cells mixed with PBS, and all mice were sacrificed after up to 2 months of injection. Once the tumor reached the maximal size allowed by institutional guidelines, mice were killed, and the tumors were dissected and weighted using a precision balance. Tumor length (L) and width (W) were monitored every second day using a caliper that was accurate to 0.5 mm. Tumor volume (V) was calculated using the following formula: $V = L \times W \times 1/2\ W$. The results are expressed as the average and standard deviation ($n = 8$ mice per condition), and statistical analysis was done using the Student's t-test. The invasive phenotype was investigated macroscopically and by histological examination. Tongue cancer and lung metastasis were fixed in formalin and embedded in paraffin. Sections with 3 μm were used for IHC (anti-NDRG1, anti-vimentin and anti-E-cadherin) as described previously.

Statistical analysis

For frequency analysis in contingency tables, statistical analyses of associations between variables were performed using chi-square test or Fisher's exact test (with significance set at $P < 0.05$). For continuous variables, the non-parametric Mann–Whitney U test was used. The disease-specific survival was defined as the interval between the beginning of treatment (surgery) and the date of death of patient or the last information for censored observations. The disease-free survival was defined as the interval between the beginning of treatment (surgery) and the date of local or regional recurrence of the tumor or the last information for censored observations. Disease-specific and disease-free survival probabilities were estimated by the Kaplan–Meier method, and the log-rank test was applied to assess the significance of differences among actuarial survival curves with a 95% confidence interval. Statistical analyses were performed using STATA® (STATA Corp., College Station, TX).

TCGA database analysis

Head and neck squamous cell carcinoma dabatases from The Cancer Genome Atlas (TCGA) (https://tcga-data.nci.nih.gov) were selected based on tumor and clinicopathological characteristics available. Data from 279 characterized patients (25) were used to construct the survival curves (Kaplan–Meir method, log-rank test) and to perform the confirmatory analysis.

Protein interactome and enrichment analyses

The protein interactions were analyzed using esyN (http://www.esyn.org) (26) by submitting NDRG1, NDRG2, NDRG3 and NDRG4 as seeds and using genetic and physical as interaction types as parameter. To search for the biological processes or molecular function, genes from the network was submitted to the gene set enrichment analysis (27) within the 50 hallmark gene sets from Molecular Signatures Database (MSigDB) (28).

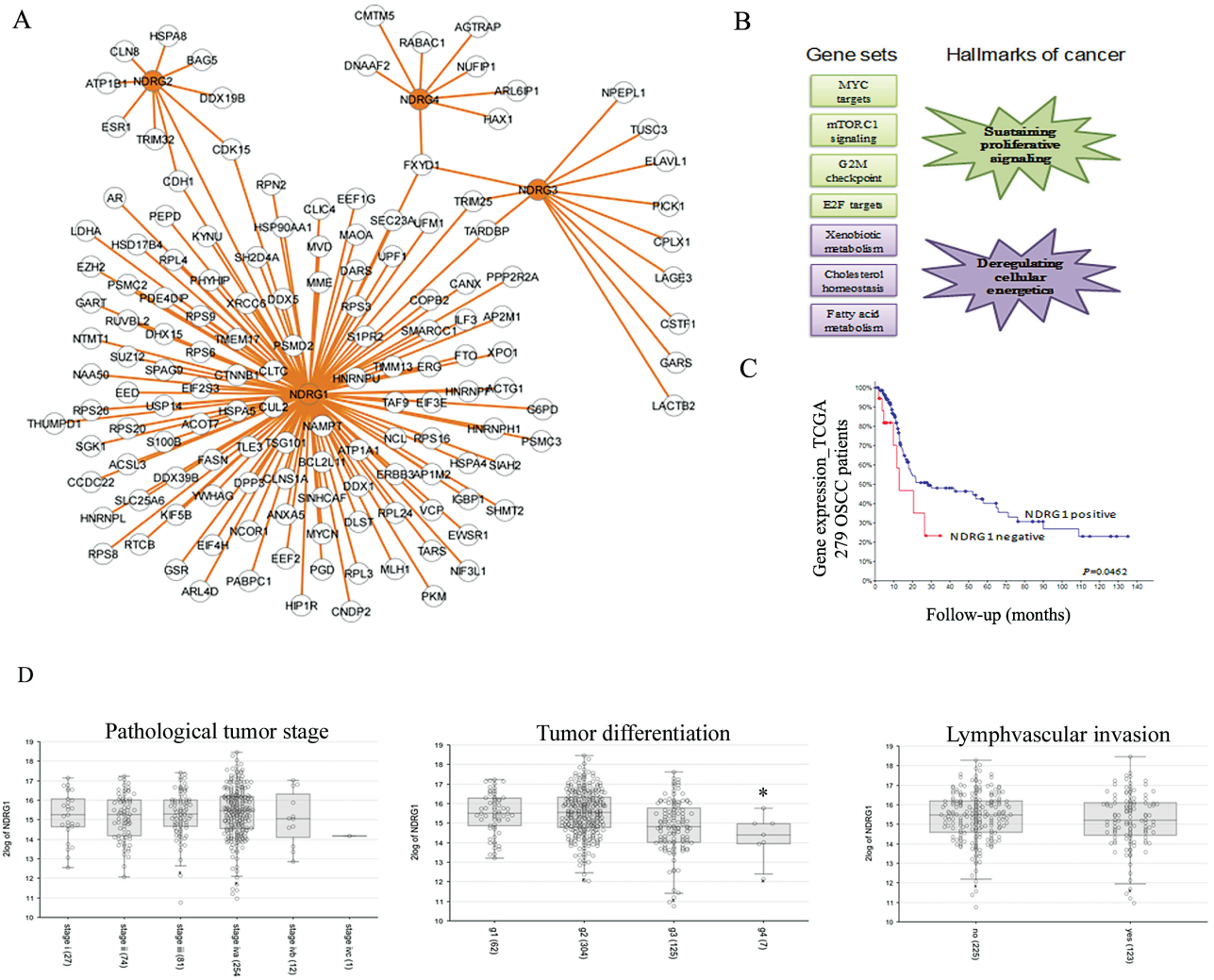

Figure 1. Genomic network analysis showing the *NDRG1* alteration involved in metastatic process. Note that the analysis involving the hallmarks of cancer showed a tight relationship with EMT-related genes, such as E-cadherin (*CDH1*), B-catenin (*CTNNB1*), MYC (*MYCN*), PTEN and heat shock proteins (*HSPs*). The overall survival analysis (Kaplan–Meir method) using the TCGA data set for 279 patients with head and neck squamous cell carcinoma revealed that downregulation of *NDRG1* had significant impact in the prognosis (log-rank test, P = 0.0462).

Result

NDRG genes are involved with mechanism of cancer development

To explore the NDRG family, the four genes (NDRG1-4) were uploaded in esyN (Easy Networks) (26) to build a gene interaction network using genetics or physical parameters, comprised additional 149 genes (Figure 1A). Enrichment analysis using gene set enrichment analysis (27) within the 50 hallmark gene sets from Molecular Signatures Database (MSigDB) (28) revealed three main biological process (false discovery rate < 10–8) involved with cell proliferation and signaling: MYC targets (14 genes), mTORC1 signaling (12 genes) and G2M checkpoint (11 genes), pointing to the involvement of this network in general mechanisms of cancer development (Figure 1B). Survival analysis using 279 patients with head and neck squamous cell carcinoma from TCGA data set showed significant lower survival probability in patients with *NDRG1* downregulation (log-rank test, *P* = 0.0462) (Figure 1C). The analysis on TCGA database considering different squamous cell carcinoma in head and neck (including oral cavity, oropharyngeal and laryngeal tumors) from 520 patients also showed that the deficiency of NDRG expression was related to advanced pathological tumor stage, low tumor differentiation and the presence of lymphovascular invasion (*P* < 0.05) (Figure 1D).

NDRG1 downregulation is associated with poor clinical outcomes in OSCC

NDRG1 was expressed in the cytoplasm of 48.9% of the OSCC patients and weak or absent in 51.1% of the cases (Figure 2A). The distribution of immunohistochemical characteristics in accordance with NDRG1 staining is summarized in Table 2. Briefly, negative NDRG1 expression in tumor samples was associated with adverse clinicopathological characteristics: patients showing positive nodal disease (*P* = 0.000789), perineural infiltration (*P* = 0.000374) and lymph-vascular invasion (*P* < 0.00001) (Figure 2B). Relative mRNA levels also confirmed that *NDRG1* was downregulated in patients with tumors in advanced clinical stages (III+IV) (*P* < 0.05) (Figure 2B). Thus, we assessed the clinical impact of NDRG1 on metastasis and survival outcome. Kaplan–Meier analysis showed that tumors lacking expression of NDRG1 have significantly poorer disease-free survival than

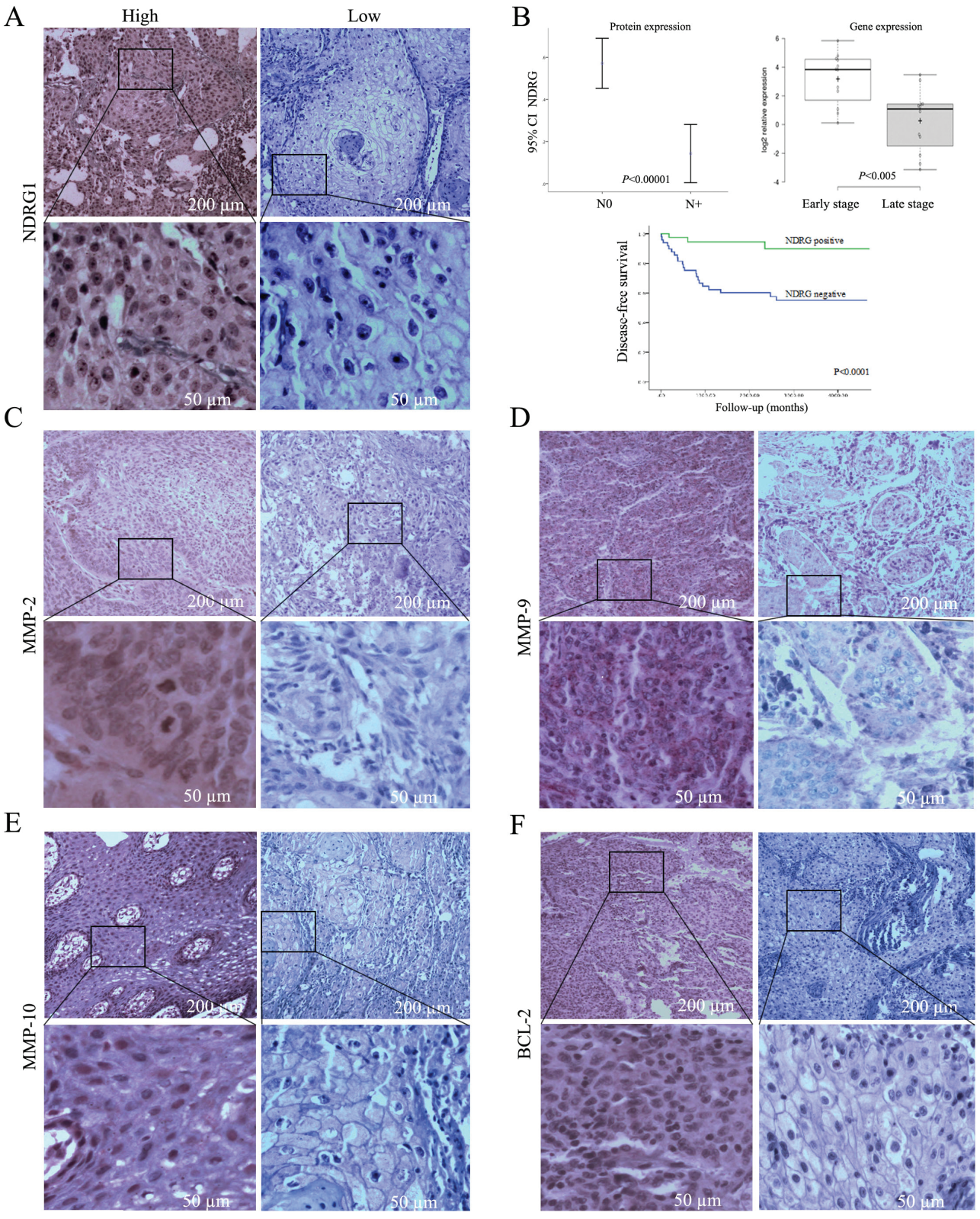

Figure 2. (**A**) Representative images of immunohistochemical staining of NDRG1, in human oral squamous cell carcinoma (OSCC). Magnification: 40×. (**B**) Distribution of NDRG1 in cytoplasmic fractions: confidence intervals (95%) show normalized mean intensity value units of NDRG1 as determined by quantitative evaluation of IHC. The *y*-axis represents numerical values corresponding to the intensity. N0: lymph nodes pathologically negative and N+: lymph nodes pathologically positive. Box plots demonstrating relative mRNA levels of *NDRG1* measured by qRT-PCR (normalized on *GAPDH* expression). Expression of mRNA *NDRG1* was downregulated in patients with tumors in advanced clinical stages (III+IV) ($P < 0.05$). The graph in the bottom shows the disease-free survival according to NDRG1 staining (Kaplan–Meier). Tumors showing positive staining for NDRG had a significantly better disease-free survival (log-rank test, $P < 0.0001$). (**C**) Immunostaining for MMP-2, (**D**) MMP-9, (**E**) MMP-12 and (**F**) BCL-2 expression were correlated to NDRG1.

Table 2. Correlation between NDRG1 immunohistochemical staining and clinicopathological characteristics in patients with oral cancer

		NDRG1		
Variable	Category	Negative	Positive	P value
Age (median)	<55 years old	3 (10.3)	26 (89.7)	0.067
	≥55 years old	7 (30.4)	16 (69.6)	
Gender	Male	5 (16.1)	26 (83.9)	0.490
	Female	5 (23.8)	16 (76.2)	
Smoking habit	No	6 (26.1)	17 (73.9)	0.264
	Yes	4 (13.8)	25 (86.2)	
Alcohol consumption	No	4 (26.7)	11 (73.3)	0.386
	Yes	6 (16.2)	31 (83.8)	
T category	T1 + T2	4 (13.3)	26 (86.7)	0.207
	T3 + T4	6 (27.3)	16 (72.7)	
N category	N0	2 (5.8)	32 (94.2)	0.000789
	N+	8 (44.4)	10 (55.6)	
Perineural infiltration	No	1 (3.2)	30 (96.8)	0.000374
	Yes	9 (42.9)	12 (57.1)	
Lymphovascular invasion	No	2 (5.1)	37 (94.9)	<0.00001
	Yes	8 (61.5)	5 (38.5)	

T, tumor size; N, lymph node involvement.

tumors with NDRG1 overexpression (log-rank test, $P < 0.001$) (Figure 2B). Loss of NDRG1 expression was associated with positive staining for MMP-2, -9 and -10 ($P = 0.022$, $P = 0.002$, $P = 0.042$, respectively), and BCL2 ($P = 0.035$), but not for MMP-1, -12 and -13 ($P > 0.05$). MMP-2, -9 and -10 were confined mostly to the peritumoral stromal tissues rather than tumor cells and found positive in 50%, 54% and 71%, respectively (Figure 2C–F).

EGF-induced NDRG1 downexpression regulates OSCC invasion through EMT promotion

The association between NDRG1 downregulation and metastatic process was investigated in preclinical models by exploring the activation of EGFR signaling in two human OSCC cell lines (HSC3 and SCC25). Exposure of OSCC cells to EGF induced a clear NDRG1 downexpression and morphological changes in almost 100% of the population as well as increased cell motility and invasion capacity (Figure 3A and B). This coincided with alteration of markers including vimentin and B-catenin overexpression and downregulation of E-cadherin and N-cadherin (Figure 3C), suggesting an intricate relation between NDRG1 expression and EMT.

The impact of NDRG1 on OSCC metastatic process was also assessed in immunocompetent syngeneic orthotopic male Balb/c mice, using the highly invasive murine OSCC cell line AT84. We selected this cell line as a model to study the interaction between oral cancer and tumor microenvironment. We evaluated the protein expression profile related to EMT process (Figure 3D) to confirm in preclinical murine model that EGF-stimulated cells also decrease NDRG1 expression and it is associated with poorly differentiated disease (Figure 3E) to promote increase in tumor migration and invasion (Figure 3F). Then, we further evaluated the effect of NDRG1 on metastatic process *in vivo*. Primary tumor overexpressing EGF showed downexpression of NDRG1 and increased number of lung metastasis compared with the control cells. *In vivo*, mice that were implanted with cells downexpressing NDRG1 had shorter survival because of fast growing tumors (Figure 3F) and respiratory problems caused by lung metastatic lesions, which were observed in >50% of animals through pathologic examination. No macroscopic lung metastasis was observed in NDRG1-positive cells, whereas the mice downexpressing the protein had to be killed due to the lung metastatic lesions before completing 2 months. As expected, an immunohistochemical panel showed the downregulation of E-cadherin (Figure 3G) and the acquisition of mesenchymal markers including vimentin (Figure 3H) and B-catenin (Figure 3I) in the group with NDRG1 downexpressed, where the cells showed invasive capacities. These results suggest that NDRG1 is essential for OSCC metastasis.

Discussion

OSCC is an aggressive disease, showing high propensity to lymphatic spread. As the occurrence of regional disease is the single most important prognostic factor in OSCC (1), it is crucial to be able to predict its occurrence. Based on molecular profiling data from the public databases, we identified NDRG1 as a potential candidate gene to be investigated specially because of the contradictory results in literature (Table 3). Of interest, the genomic network mapping we created contained, along with NDRG1, a network of known genes with prognostic value in metastatic OSCC, such as E-cadherin, N-cadherin, B-catenin, heat shock proteins and Rab-GTPase (23,24,29). This confirms that our approach is valid, as there is a substantial overlap with already recognized genes involved with tumor progression and metastatic process. Exploring genes from NDGR family may unveil the mechanisms related to EMT, and metastasis formation, that all these genes are involved with.

Once NDRG1 was identified, we evaluated the prognostic impact in an independent set of patients at gene and protein levels. The negative or weak expression of NDRG1 was associated with occurrence of nodal disease, advanced clinical stage, adverse clinicopathological features and worse disease-free survivals in Kaplan–Meier analysis. In other cancers, the prognostic importance of NDRG1 has been widely reported (Table 3); meanwhile, only one study has come to similar conclusions in OSCC. Interestingly, NRDG1 seems to act as a metastasis suppressor in numerous cancers such as breast, brain, pancreatic, colorectal, prostate and oral cancer (15,30–34). However, in hepatocellular carcinoma, high NDRG1 expression was associated with poor prognosis (35).

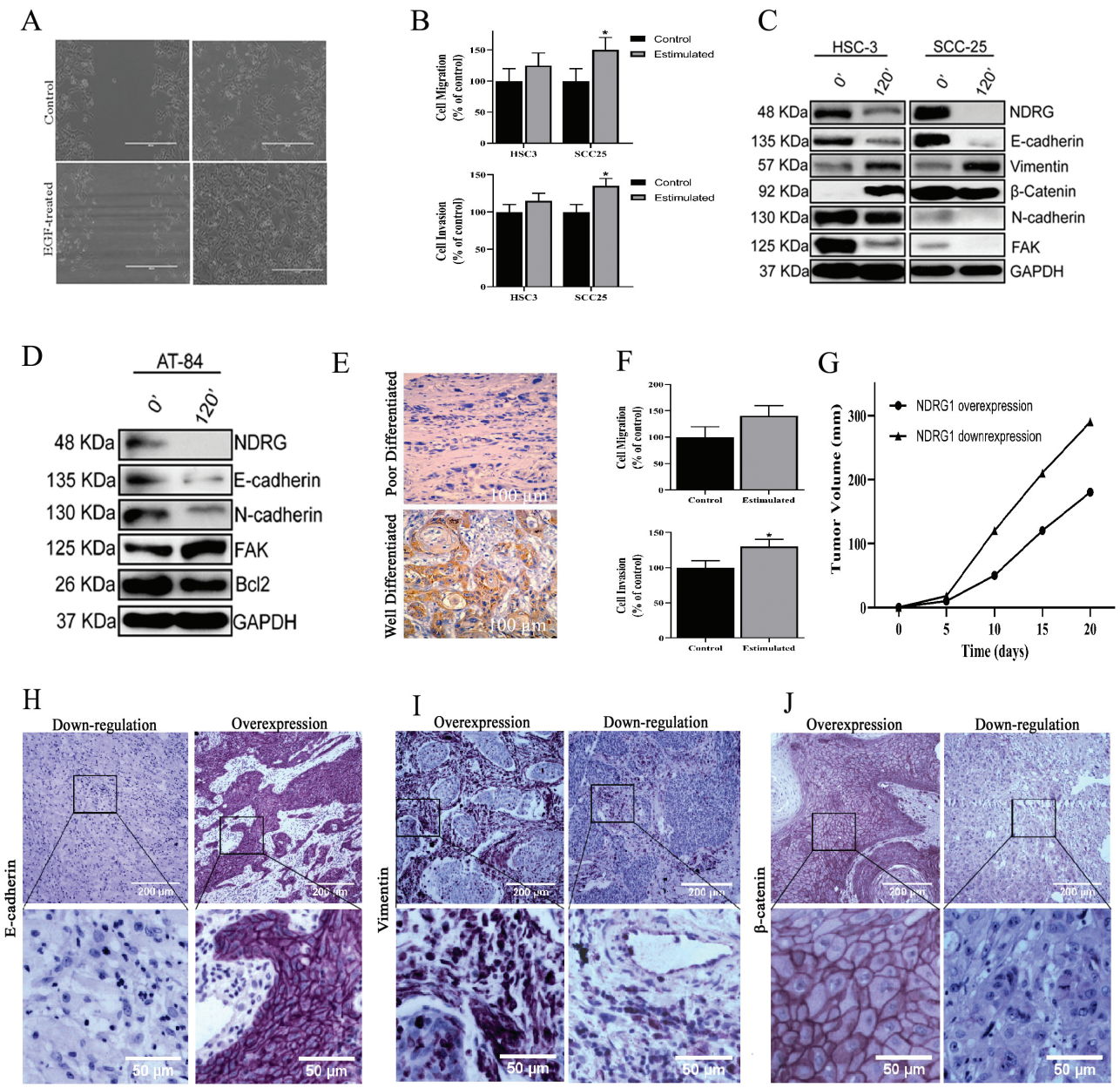

Figure 3. (**A**) Epithelial growth factor (EGF)-driven epithelial–mesenchymal transition (EMT) was able to change the morphology to fibroblast like in preclinical models. (**B**) A migration and invasion assay was conducted before and after EGF treatment using the Boyden chamber assay. The bar graph represents the mean number (±standard error [SE]) of invaded cells ($P < 0.005$). (**C**) Western-blot analysis confirmed reduced expression of NDRG1 and EMT activation in human oral cancer cell lines (HSC-3, SCC-25) and (**D**) in the murine cell line (AT84). Protein expression was measured at baseline (0') and 2 h after EGF treatment (120'). GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase and it was used as control. (**E**) Immunohistochemical staining of NDRG1 showed the protein downexpressed in poorly differentiated—grade III OSCC and overexpression in well-differentiated tumors. (**F**) Cell migration and invasion were measured and confirmed that the downregulation of NDRG1 increase motility. (**G**) Tumor weights are illustrated at the time mice were killed ($n = 8$) from animals implanted with AT84 control and AT84 EGF-stimulated cells implanted orthotopically in the tongue. (**H**) *In vivo* mouse model reveals NDRG1 immunostaining was significantly reduced in the primary tumor from the metastatic models; in another way, it was observed an overexpression of vimentin and B-catenin protein expression.

We pursued our investigation to understand the molecular impact of NDRG1 downexpression in the EMT phenotype. We simulated activation of EGFR cascade in a preclinical model in human and mice OSCC cells lines. EGFR is a therapeutic target in OSCC, as the chimeric anti-EGFR antibody cetuximab is now part of clinical routine in the treatment of metastatic OSCC (36). EGF stimulation resulted in a dramatic downexpression of *NDRG1*. In our animal model, NDRG1 downexpression was associated with lung metastases. In agreement with our data, Lee *et al*. showed that NDRG1 knockdown enhanced tumorigenesis in a mouse xenograft animal and increased the incidence of lung metastasis (15). In our study, the downregulation of NDRG1 resulted in activation of classical EMT markers (37,38). NDRG1 is thought to directly interact with the catalytic domains of MMP-2 and MMP-9 and hence increase cellular invasive properties (15). Furthermore, NDRG1-mediated EMT activation occurred through upregulation of the E-cadherin expression and downregulation of the N-cadherin, Vimentin, Snail-1 and Slug

Table 3. Molecular features, expression and function of the human NDRG1 in different types of cancer

Gene ID	Aliases	Chromosome location	Protein length	Type of cancer	Expression
10397	GC4 RTP DRG1 NDR1 NMSL TDD5 CAP43 CMT4D DRG-1 HMSNL RIT42 TARG1 PROXY1	8q24	394	Oral	NDRG1 expression lead downregulation of EMT, MMP-2 and MMP-9 in human OSCC cells (15).
				Breast	NDRG1 expression is reduced in patients with lymph node or bone metastasis. High expression is associated with good prognosis. NRDG1 used as indicator of the therapeutic efficacy of anti-estrogenic agents (29).
				Prostate	NDRG1 expression is reduced in patients with lymph node or bone metastasis compared with those with localized prostate cancer. Expression is inversely correlated with Gleason grading and overall survival (30).
				Colorectal	NDRG1 inhibits 'stemness' of colorectal cancer via downregulation of nuclear B-catenin and CD44 (31).
				Pancreas	NDRG1 loss of expression increased invasion, tumor growth and angiogenesis (32)
				Brain	Decreased expression of NDRG1 in glioma related to tumor progression and poor survival of patients (33)
				Liver	NDRG1 expression was associated with vascular invasion, metastasis and shorter overall survival (34).

(15,24). NDRG1 expression inhibits the Wnt signaling cascade. The activation of the Wnt-B-catenin pathway has been shown to be of critical importance in metastatic progression rather than initial tumorigenesis, as it has little effect on proliferation and viability of cancer cells, but it significantly promotes metastatic phenotypes through induction of stemness properties such as self-renewal and metastatic niche (14). Notably, the NDRG1-mediated Wnt suppression restored adhesion complex between cells whose integrity is essential for epithelial cell differentiation and polarity. Restoring the expression of NDRG1 is therefore an attractive option for therapeutic target. A small molecule (novel anti-tumor agents of the di-2-pyridylketone class of thiosemicarbazones, namely di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone and di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone, which markedly upregulate NDRG1) has shown the capability to induce restoration of NDRG1 expression, which led in a preclinical model to suppressed metastasis to the lymph nodes and to distant sites (39).

In conclusion, NDRG1 downexpression can serve as a potential predictor of metastasis in oral cancer. Downregulation of NDRG1 is associated with acquisition of metastatic abilities. Interfering with NDRG1 and its upstream proteins may result in rescue of NDRG function as potential therapeutic strategy to prevent oral cancer progression to metastasis.

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