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Diabetes Increases both N-ras and Ets-1 Expression During Rat Oral Oncogenesis Resulting in Enhanced Cell Proliferation and Metastatic Potential

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Abstract. *Background: The expression of N-ras and ets-1 proteins was investigated in an experimental model of chemically-induced carcinogenesis in normal and diabetic (type I) Sprague-Dawley rats. Materials and Methods: Tissue sections ranging from normal mucosa to moderately-differentiated oral squamous cell carcinoma were studied using monoclonal antibodies against N-ras and ets-1 proteins. Results: In diabetic rats, N-ras expression increased with tumor advancement, while in normal rats N-ras was not detected in initial stages of oral oncogenesis and increased only in well-differentiated OSCC. The same pattern of elevated ets-1 expression was observed both in diabetic and normal rats, but in cancerous stages this expression was higher in diabetic than in normal rats. Conclusion: It seems that diabetes may contribute to increased cell proliferation due to N-ras constitutive activation, as well as to enhanced invasion and metastatic potential by increasing ets-1 levels.*

Oral squamous cell carcinoma (OSCC), including the oral cavity and oropharynx, constitutes the sixth most common cancer worldwide (1). The prognosis for OSCC remains dismal, despite the recent advances in surgery, radiation and chemotherapy, especially if malignant tumors are not diagnosed during the early stages of cancer formation (2). Oral oncogenesis is a multistep process including aberrant expression of oncogenes and tumor suppressor genes leading to the disruption of the normal pathways which control the basic cellular functions, such as cell proliferation and differentiation (3).

N-ras controls differentiation or cell proliferation *via* a large network of signaling pathways. Following its activation, N-ras generates the MAPK cascade leading to the activation of the extracellular signal regulated kinases 1 and 2 (ERK1 and 2), which in turn leads to phosphorylation and activation of transcription factors resulting in the switching on of a number of genes associated with proliferation (4, 5). The amplification of *N-ras* gene and certain *N-ras* mutations (especially these occurring in codons 61, 12, or 13) produce constitutively active proteins which have been implicated in oncogenesis and occur in 30% of all types of human cancer (5-7).

The *c-ets-1* proto-oncogene (E26 transformation specific-1) encodes for a transcription factor which is involved in the transcriptional regulation of several genes implicated in tumor invasion and metastasis, such as collagenase I, stromelysin, and the urokinase plasminogen activator (8-10). Growth-factor and stress-activated signaling pathways act through small GTP-binding proteins, such as Ras, stimulating down-stream kinases that ultimately activate MAP kinases (11). MAPK activate ets-1 by phosphorylation, which in turn regulates specific genes (11). The most frequent changes in ets-1 activity which contribute to several types of cancer include misexpression of hyperactive ets-1 fusion proteins due to reciprocal chromosomal translocations, as well as overexpression of normal proteins (12).

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There is scarce literature regarding the expression of *N-ras* protein in OSCC (13-15). In addition, dysregulation of the *N-ras* gene is a rare event in the progression of oral cancer suggesting that alterations in N-ras may not play a major role in oral squamous carcinogenesis (16-18). Ets-1 has been implicated in human OSCC and ets-1 levels seem to correlate well with the grade of invasiveness and metastasis (19-21). Our group investigated N-ras and ets-1 expression in a model of sequential histological stages in the oral region in hamsters and suggested that neither N-ras nor H-ras affects ets-1 expression; therefore, the pathway implicating them might be altered during oral oncogenesis (22).

Several metabolic and immunological changes which affect oral mucosa and are associated with a variety of oral conditions can be attributed to diabetes mellitus (23). It has been well-documented that diabetes are associated with a variety of oral and periodontal conditions, such as alterations in salivary secretions, dental caries, glossitis and oral lichen planus (24). The first study investigating a molecular basis for the association between oral cancer and diabetes (type I) in a rat animal model was published by our group (25).

Therefore, we used the same experimental model of diabetic (type I) and normal rats with induced OSCC to examine the expression of N-ras and ets-1 in normal oral mucosa tissues and in sequential stages of tumor formation, in order to examine the equivalence with the hamster model and the effect of diabetes in the N-ras/ets-1 pathway.

Materials and Methods

Thirty-seven female Sprague-Dawley rats purchased from the Hellenic Pasteur Institute (Athens, Greece) at the age of six weeks, weighing approximately 135 g each, were used in this study. The rats were handled in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

The animals were randomly divided into four groups: i) Group D (n=6): diabetic rats without carcinogenesis; ii) Group Dc (n=13): diabetic rats used for induced carcinogenesis; iii) Group N (n=6): normal rats without carcinogenesis; iv) Group Nc (n=12): normal rats used for induced carcinogenesis.

The induction of diabetes was performed in 19 previously overnight fasted animals by a single intraperitoneal injection of streptozotocin (STZ) dissolved in saline buffer at a dose of 70 mg/kg of weight (ZANOSAR; Pharmacia & Upjohn Co., USA) and determined by glucose levels in blood after three weeks, as described elsewhere (25). Oral cancer was induced in Dc and Nc animals by the application of the carcinogen 4-nitroquinoline N-oxide (4NQO) at a concentration of 5% in propylene glycol 3 times per week for 5 months to the rats' hard palate (Fluca AG Chemische Fabrik, Switzerland), as described elsewhere (25). Clinical signs of oral lesions putatively tumor-related were observed within 6 months after the last application of carcinogen. After sacrifice of animals by ether treatment, the oral regions with cancer (mainly the palate and tongue) of Dc and Nc rats and the respective regions of D and N rats were excised for immunohisto-chemical analysis (25).

Pathological evaluation. The histological status of the lesions was defined after examination of the complete section under light microscopy and the tissue profiles were classified into the following categories: normal mucosa, hyperplasia, dysplasia, early invasion, well- and moderately-differentiated carcinoma. In every sample, all possible different lesions were evaluated.

Immunohistochemical analysis. Surgical specimens were fixed in 10% neutralized formaldehyde solution and embedded in paraffin. Three sections of 4 μm thickness were prepared from each specimen and were mounted on Super Frost Plus-coated glass slides (Menzel and Co., Braunschweig, Germany). One section was stained with hematoxylin and eosin for routine histological evaluation, while the other two were used for immunohistochemical detection of N-ras and ets-1 proteins as described elsewhere (26) with monoclonal primary antibodies against N-ras (c-N-ras, Ab-1, CatOP25, diluted 1:100; Oncogene Research Products, San Diego, CA, USA) and ets-1 (ets-1 Ab-1, Clone 1G11, diluted 1:10; Lab Vision Corporation, CA, USA). Each section was studied immunohistochemically and various representative histological regions in each section were analysed.

Human brain and tonsils which strongly express N-ras and ets-1 respectively were used as positive controls. Negative controls of human brain and tonsils were processed in the same manner, using PBS instead of the primary antibody. All slides were independently reviewed by two investigators blindly. The consecutive hematoxylin-eosin-stained slides were evaluated by a pathologist experienced in oral pathology, without knowing the N-ras and ets-1 staining patterns.

Results of N-ras or ets-1 expression were presented as the percentage of positively-stained cells in each sample.

Statistical analysis. For each animal, we evaluated the percentages of N-ras and ets-1 expression in all possible lesions. All lesions were accumulated and classified into the following categories: "normal mucosa, hyperplasia, dysplasia, early invasion, well- and moderately differentiated carcinoma". Statistical analyses were performed using the two-tailed Student's *t*-test for each group of animals and each histological category. The percentage of positively-stained cells from each non cancerous or precancerous condition (hyperplasia, dysplasia) were compared to normal tissue, while the percentage of positively-stained cells from each tumor (early invasion, well- and moderately-differentiated carcinoma) were compared to the mean value of percentages of the non-cancerous and precancerous conditions. In all cases with no normal distribution, the results of both the Wilcoxon test and two-tailed Student's *t*-test provided the same level of significance.

Results

The histological status of biopsies observed in various regions of rats from all groups (N, Nc, D, Dc) after induced oral oncogenesis is summarized in Table I. The experimental model seems valid since several normal, non-cancerous, precancerous and cancerous regions in the various tissue biopsies were collected and further analysis of immunostaining data was implemented (Figures 1, 2). In all cases with no normal distribution, the results of both the Wilcoxon test and two-tailed Student's *t*-test provided the same level of significance.

Table I. *Histological status of biopsies in groups N, D, Nc and Dc.*

	Number of rats			
	Control groups		Experimental groups	
	(N)	(D)	(Nc)	(Dc)
Normal tissue	3	5		
Hyperplasia	3	1		1
Dysplasia			1	4
Early invasion			1	1
Well-differentiated carcinoma			6	4
Moderately-differentiated carcinoma			4	3

The percentages of positive expression of N-ras and ets-1, in the various histological categories for non-diabetic animals from groups N and Nc are shown in Table II. N-ras immunoexpression was found negative during the stages ranging from normal mucosa to early invasion. Statistical analysis revealed a significant increase in N-ras levels in well differentiated carcinoma when compared to the mean percentages of the non-cancerous and precancerous conditions ($p<0.05$) which remained relatively stable in moderately differentiated OSCC. The analysis of ets-1 expression revealed no statistically significant results. Nevertheless, it was observed that ets-1 immunoexpression was much higher in precancerous stages (hyperplasia, dysplasia), lower in the stage of early invasion and higher again in well differentiated OSCC.

Table III summarizes the percentages of positively stained cells for N-ras and ets-1 in the various histological categories for diabetic animals from groups D and Dc. In the precancerous stages, N-ras was elevated significantly compared to normal oral mucosa ($p<0.001$), lower in well differentiated OSCC and higher again in the last stage of moderately differentiated OSCC but not significantly. The comparison of ets-1 positive percentages between each non-cancerous and precancerous stage with normal oral mucosa revealed a statistical difference for hyperplasia. In addition, all tumor stages in the comparison between the mean percentages of non-cancerous and precancerous conditions revealed significantly higher ets-1 protein levels.

Figure 3a and b show the expression of N-ras and ets-1 in the different tissue categories in normal and diabetic rats. In diabetic rats, N-ras progressively increased with the exception of well differentiated OSCC where a non-significant decrease was noted. On the contrary, in normal rats without diabetes, N-ras was found higher only in moderately differentiated OSCC. Regarding ets-1, the same pattern of elevated expression was observed after the formation of well differentiated OSCC and was higher in diabetic than in normal rats.

Discussion

This study is, to our knowledge, the first investigating the expression of N-ras and ets-1 in an experimental animal system of chemically induced oral carcinogenesis in normal and diabetic rats. Although theoretically STZ depresses cell-mediated immune responses (27), no growth advantage of tumors was observed in Dc rats *versus* Nc rats. Based on these data, diabetes does not seem to confer an earlier and more aggressive effect on carcinogenesis in the oral region, if a carcinogen is continuously applied for five months. Nevertheless, this robust approach of induced carcinogenesis might mask a subtler increase in cancer susceptibility of diabetic *versus* normal rats. A previous similar experimental study by our group implicated diabetes mellitus as a risk factor for the development of OSCC (25). In accordance with our previous findings, some epidemiological studies have associated diabetes with oral precancerous and cancerous lesions (28-30).

In the present study, an experimental system of chemically induced diabetes mellitus (type I) in rats was used and its effect in the N-ras/ets-1 pathway on oral carcinogenesis was investigated. The status of induced diabetes was established based on higher glucose levels and decreased body weight detected in diabetic rats compared to normal controls. As previously described, there were no significant differences in the histological status of oral cancer biopsies obtained from diabetic and normal rats after carcinogen treatment for a prolonged period of five months (25).

The N-ras and ets-1 expression was investigated in sequential stages of oral oncogenesis, varying from normal oral mucosa to moderately-differentiated oral carcinomas, obtained from normal and diabetic rats with or without induced oral cancer. In diabetic rats, N-ras was progressively higher in comparison to normal rats without diabetes, in which N-ras expression was negative in the initial stages of oral oncogenesis and higher only in well differentiated OSCC. On the other hand, practically the same pattern of elevated ets-1 expression was observed both in diabetic and normal rats, but in cancer stages this expression was higher in diabetic than in normal rats.

The observed increase of N-ras expression in diabetic animals may be justified *via* the insulin signalling pathway. The insulin signalling pathway includes a series of phosphorylation cascades linking initial activation of the insulin receptor (IR) to downstream substrates, such as IRS-1 (25). Activation of IRS-1 promotes Grb2/SOS complex binding which results in the activation of N-ras, leading to the ras/raf/MAP kinase cascade (31). In

Table II. *Percentage of N-ras- and ets-1-positive cells in the various tissue categories for normal rats (N and Nc).*

		Non-cancerous and precancerous		Tumour		
	Normal oral mucosa	Oral mucosal hyperplasia	Oral mucosal dysplasia	Early invasion	Well-differentiated OSCC	Moderately-differentiated OSCC
N-ras						
Mean percentage	0 (N=4)	0 (N=9)	0 (N=4)	0 (N=4)	1.67 (N=6)	2.5 (N=4)
Mean non-cancerous and precancerous condition		0				
Probability of *t*-test		N.S.[a]	N.S.[a]	N.S.[a]	$p<0.05$	N.S.[a]
ets-1						
Mean percentage	0 (N=7)	1.3 (N=13)	1.5 (N=8)	0 (N=2)	2.3 (N=3)	1.7 (N=7)
Mean non-cancerous and precancerous condition		1.4				
Probability of *t*-test		N.S.[a]	N.S.[a]	N.S.[a]	N.S.[a]	N.S.[a]

[a]N.S: No statistical difference.

Table III. *Percentage of N-ras- and ets-1-positive cells in the various tissue categories for diabetic rats (D and Dc).*

		Non-cancerous and precancerous		Tumour		
	Normal oral mucosa	Oral mucosal hyperplasia	Oral mucosal dysplasia	Early invasion	Well-differentiated OSCC	Moderately-differentiated OSCC
N-ras						
Mean percentage	0 (N=22)	2.9 (N=33)	4.3 (N=21)	6.25 (N=4)	3.9 (N=23)	10.9 (N=11)
Mean non-cancerous and precancerous condition		3.46				
Probability of *t*-test		$p=0.001$	$p<0.001$	N.S.[a]	N.S.[a]	N.S.[a]
Ets-1						
Mean percentage	0 (N=10)	1.4 (N=38)	0.3 (N=26)	1.5 (N=6)	4.4 (N=15)	3 (N=15)
Mean non-cancerous and precancerous condition		0.9				
Probability of *t*-test		$p<0.05$	N.Sa	$p<0.05$	$p<0.01$	$p<0.01$

[a]N.S: No statistical difference.

accordance with this notion, we observed increased IRS-1 expression in diabetic compared to normal animals in the same experimental system (25).

Our group had previously performed a study investigating the expression of N-ras and ets-1 in a similar experimental system in hamsters without diabetes (22). In hamsters, N-

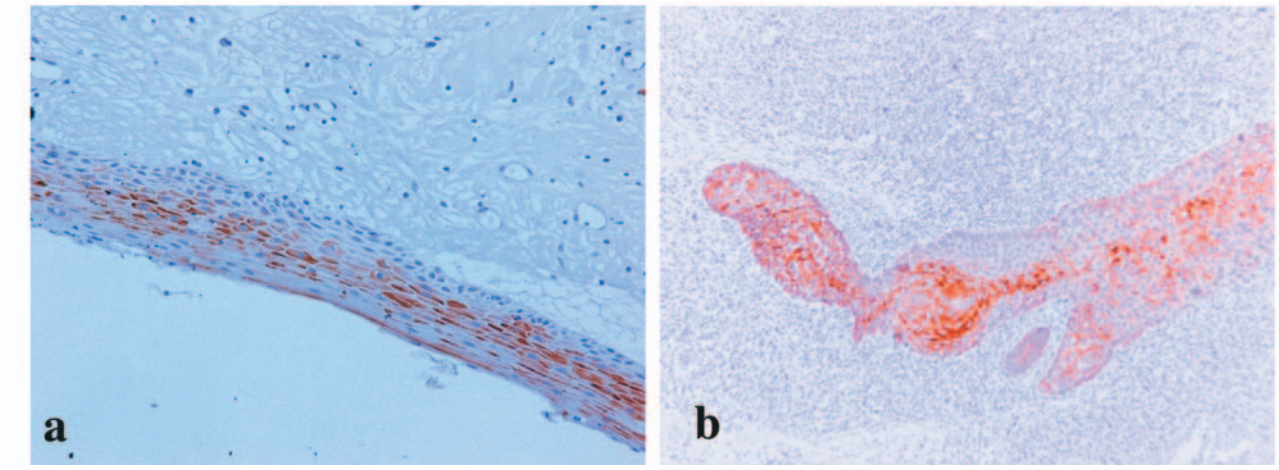

Figure 1. *(a) Evident N-ras cytoplasmic immunoreactivity in mildly dysplastic oral mucosa from diabetic rats (x200). (b) Increased N-ras immunostaining from an area of early invasion in diabetic rats (x100).*

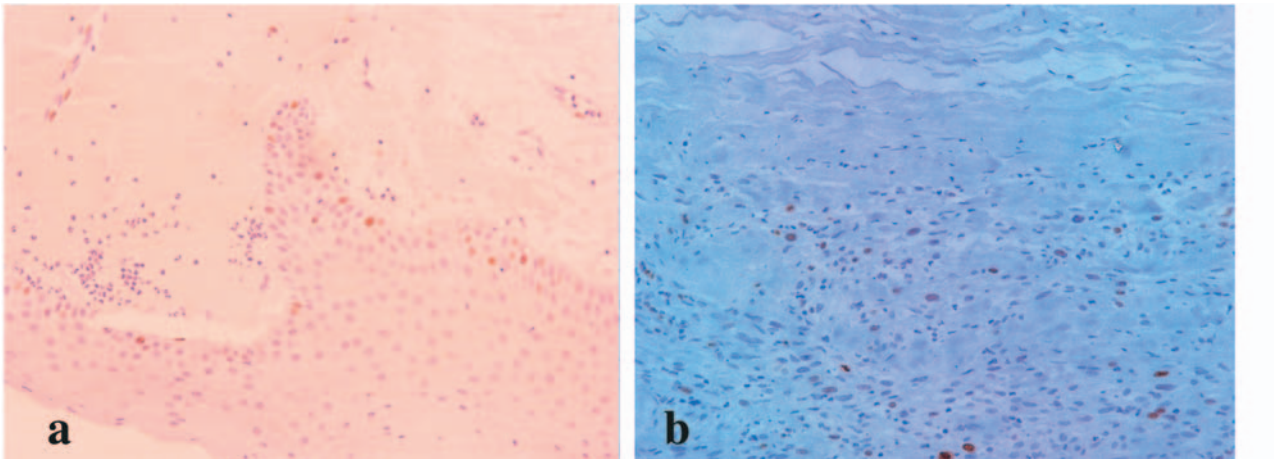

Figure 2. *(a) Nuclear ets-1 immunostaining at the invasive border of a moderately differentiated oral squamous cell carcinoma in non-diabetic rats (x200). (b) Minimal ets-1 immunoreaction in hyperplastic oral mucosa of non-diabetic rats (x200).*

ras follows a descending series of expression (Figure 4), while in rats N-ras is higher in well differentiated carcinoma (Figures 3, 4). On the other hand, neither in hamsters or in rats was ets-1 detected in normal oral mucosa (Figure 4), while higher expression was observed in the subsequent stages of oral carcinogenesis, with ets-1 values being higher in hamsters than in rats. These differences may be attributed to dissimilar physiology and diverse significance of the role of N-ras in oral oncogenesis in the two rodents.

Conclusion

The findings of this study suggest that in oral oncogenesis, the N-ras expression is not associated with ets-1 expression, contrary to other types of cancer (11). In addition, diabetes seemed to promote the elevation of both N-ras and ets-1 throughout the process of oral oncogenesis, although through different mechanisms. It seems that diabetes may contribute to increased cell proliferation and aggressiveness of cancer due to the constitutive activation of N-ras (5). Furthermore, by increasing levels of the transcription factor ets-1, diabetes may result in the up-regulation of genes associated with invasion and metastatic potential of tumor cells (32). It seems that animal models may be a most useful tool in understanding important mechanisms in oral oncogenesis.

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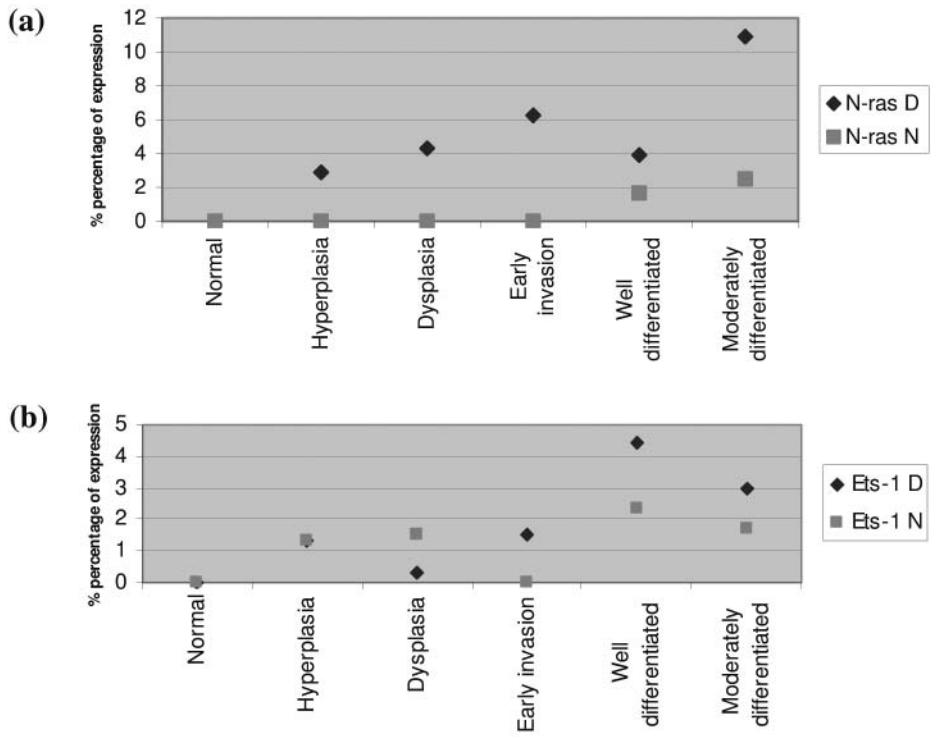

Figure 3. *Expression of (a) N-ras and (b) ets-1 in the different tissue categories in normal (N) and diabetic (D) rats.*

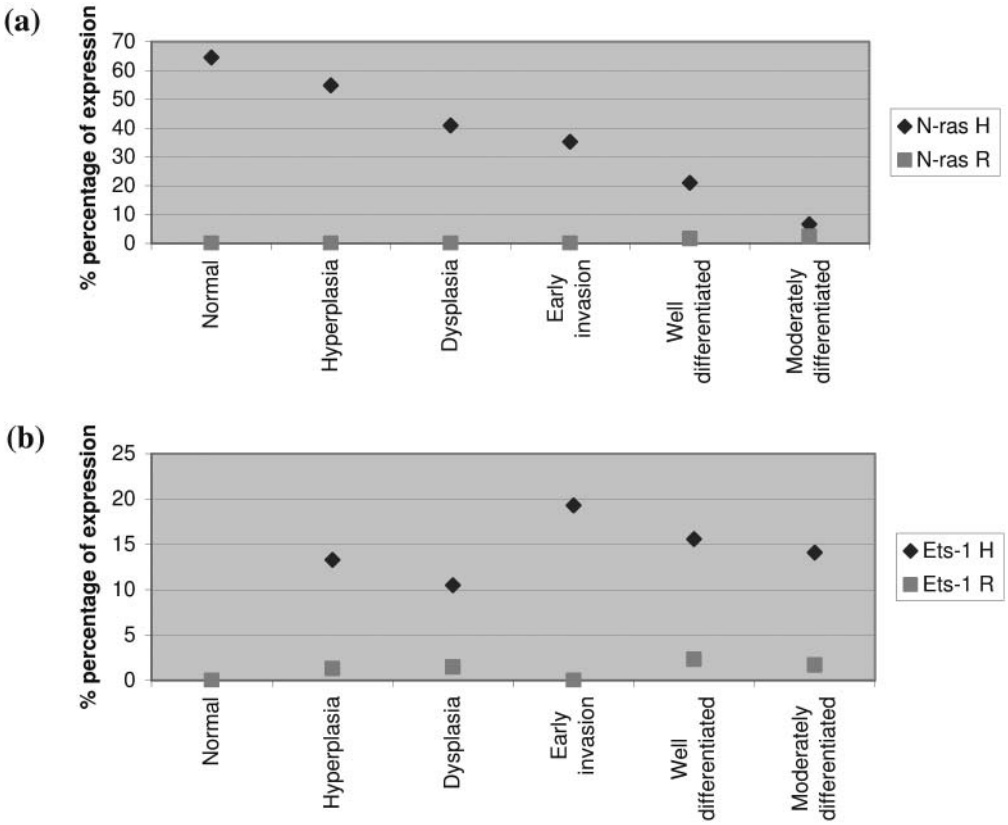

Figure 4. *Expression of (a) N-ras and (b) ets-1 in the different tissue categories in normal hamsters (Derka) (H) and normal (D) rats (this study).*

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