The immunohistochemical investigations of cadherin “switch” during epithelial-mesenchymal transition of tongue squamous cell carcinoma

MIHAI-CĂTĂLIN AFREM1), CLAUDIU MĂRGĂRITESCU2), MONICA MIHELA CRĂŢOIU3), MIRELA CIUCĂ4), CĂLIN-GABRIEL ŞARLĂ5), OVIDIU SIMION COTO5)

1)PhD student, Department of Histology, University of Medicine and Pharmacy of Craiova, Romania
2)Department of Pathology, University of Medicine and Pharmacy of Craiova, Romania
3)Department of Prosthetics and Oral Rehabilitation, University of Medicine and Pharmacy of Craiova, Romania
4)PhD student, Department of Pathology, University of Medicine and Pharmacy of Craiova, Romania
5)Department of Physical Education and Sports, "Vasile Goldiş" Western University, Arad, Romania

Abstract
Oral cancer was ranked as the sixth worldwide most common cancer, but recent studies noticed an overall downward trend in its incidence. However, for tumors localized on the tongue, the incidence seems to increase. The malignant transformation of many carcinomas is associated with loss of epithelial differentiation and gain of a mesenchymal phenotype, a process known as epithelial-mesenchymal transition (EMT) which for oral squamous cell carcinomas (OSCC) could be a predictor and a prognostic factor. The aim of our study was to investigate immunohistochemically the E-cadherin/N-cadherin “switch” and vimentin expression as markers of EMT process in tongue OSCC. Thus, we analyzed 15 cases of tongue OSCC by enzymatic double immunohistochemistry using the following double pairs of antibodies: E-cadherin/vimentin and N-cadherin/E-cadherin. E-cadherin reactivity was recorded in all the investigated cases, the pattern of expression being both membranous and cytoplasmic, with the membrane pattern decreasing simultaneously with the decrease of the differentiation degree and with the increase of invasion phenotype, while the cytoplasmic pattern had an opposite behavior. Tumor parenchyma reactivity for vimentin was noticed in 73.3% and its expression was more obvious in tumor cells from the periphery of proliferative islands and in acantholytic carcinomatous cells. N-cadherin reactivity was restricted to only 33.33% of the investigated cases and its expression was prevalent in poorly differentiated forms. In conclusion, in tongue squamous cell carcinomas at the invasion front the E-cadherin reactivity decreases while vimentin expression increases, with a cytoplasmic N-cadherin reactivity in a few of the observed cases. This EMT event in human cancers EMT [9].

Keywords: E-cadherin, epithelial-mesenchymal transition, N-cadherin, squamous cell carcinoma, tongue, vimentin.

Introduction
Oral cancer is the sixth worldwide most common cancer [1]. International Agency for Research on Cancer (IARC), in 40 European countries, in 2012 reported the age-standardized rates (ASRs) (per 100 000) by gender, for incidence of oral and pharyngeal cancer of 18.2 in men and 4.9 in women, while the reported rate of mortality was about 8.4 and respectively 1.6 [2]. For Romania, the IARC estimation was about 29.6 in men and 3.3 in women for incidence, while for death was reported a rate of 17.9 and respectively 1.6. In addition, although the overall trend in the incidence of oral squamous cell carcinoma (OSSC) is to decrease the incidence of tongue squamous cell carcinoma seems to be growing in younger individuals form white race [3].

The malignant transformation of many carcinomas is associated with loss of epithelial differentiation and gain of mesenchymal phenotype, a process known as epithelial-mesenchymal transition (EMT) [4].

Relatively recent studies showed that the presence of EMT process is a predictor of oral squamous cell carcinomas progression and also their prognostic factor [5, 6].

Generally, cells undergoing such process show reduced expression of epithelial markers, including E-cadherin, desmoplakin, cytokeratins, claudins, occluding and beta-catenin and overexpression of mesenchymal markers such as N-cadherin, vimentin, fibronectin and Snail1/2 [7, 8].

The cadherin “switches”, consisting of E-cadherin expression loss and N-cadherin expression gain is a key event in human cancers EMT [9].

Moreover, the involvement of Snail in regulation of E-cadherin expression during EMT process was first proved in OSCC model, where the cells transfected with Snail had a full TEM phenotype with fibroblast-like morphology, expression of vimentin filaments, E-cadherin/N-cadherin “switch” and without hemidesmosomes [10].

The aim of our study was to investigate immunohistochemically the E-cadherin/N-cadherin “switch” and vimentin expression as markers of EMT process in tongue OSCC.
**Materials and Methods**

Fifteen cases of tongue OSCC were selected from the archive of the Department of Pathology of the Emergency County Hospital of Craiova, Romania. The mean age of these patients was of 63 years, and the tongue edges were the most commonly involved. Eight cases were moderate differentiated, four were classified as well-differentiated and the other belonged to poor differentiated. As for their respective clinical stage, most cases were classified as stage I TNM (seven cases), the others being diagnosed as stage II (five cases) and stage III (three cases).

Five μm-thick sections were deparaffinized in xylene, rehydrated through graded alcohol series, and subjected to enzymatic double immunohistochemistry using the following double pairs of antibodies: E-cadherin (mouse anti-human, monoclonal, NCH-38, Dako, Redox, Bucharest, Romania, in dilution 1:50)/vimentin (rabbit anti-human, monoclonal, SP20, Cell Marker, Tunic, Bucharest, Romania, in dilution 1:200) and N-cadherin (mouse anti-human, monoclonal, 6G11, Dako, Redox, Bucharest, Romania, in dilution 1:50)/E-cadherin (rabbit anti-human, monoclonal, EP700Y, Cell Marker, Tunic, Bucharest, Romania, in dilution 1:50). For the first antibody developing, we used citrate pH 6 heat-induced antigen retrieval, LSAB2 System-HRP (Dako, Redox, Romania) and DAB (Dako, Redox, Romania) as chromogen, according to the manufacturing protocols. After blocking the endogenous Biotin with Avidin/Biotin Blocking Kit (Dako, Redox, Romania) as chromogen, to the manufacturing protocols. Negative controls were obtained by omitting the primary antibodies.

The assessment of tissue immunoreactivity was made semiquantitatively by counting the positive tumor cells under ×400-power magnification from five random fields and the results were expressed as percentage of immunoreactive cells from all of total tumor cells counted. Also, tumor reactivity was quantified as score 0 – absence of reactive cells from all of total tumor cells counted. Also, the reaction was present in all the investigated cases.

Statistical analysis was done in SPSS version 16.0 for Windows, using the $\chi^2$-test for dependence assessment; Student’s t-test and ANOVA testing being used for paired or multiple inter-group comparisons, all results were considered statistically significant for a $p$-value <0.05.

**Results**

### E-cadherin reactivity

Most cases (11 cases) were scored as 2, the reaction being present in all cases. Because the tumor immunoreactivity was noticed both at membrane and cytoplasmic level, we separately assessed the number of tumor cells that were only membrane stained and respective those tumor cells that present only cytoplasmic reaction and then we tried to correlate these scores with the main morphological parameters (TNM stage, tumor differentiation degree and type of invasion pattern) (Table 1).

<table>
<thead>
<tr>
<th>Morphological parameter</th>
<th>Subcategories</th>
<th>Membrane E-cadherin reactivity (mean ± SD)</th>
<th>Cytoplasmic E-cadherin reactivity (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNM stage</td>
<td>I (n=5)</td>
<td>41.23±36.32</td>
<td>15.43±15.21</td>
</tr>
<tr>
<td></td>
<td>II (n=3)</td>
<td>39.45±28.31</td>
<td>17.23±16.57</td>
</tr>
<tr>
<td></td>
<td>III (n=4)</td>
<td>20.34±23.52</td>
<td>18.73±24.76</td>
</tr>
<tr>
<td>Differentiation degree</td>
<td>Well (n=4)</td>
<td>55.73±31.53</td>
<td>25.37±21.63</td>
</tr>
<tr>
<td></td>
<td>Moderate (n=8)</td>
<td>37.43±29.12</td>
<td>16.31±18.72</td>
</tr>
<tr>
<td></td>
<td>Poor (n=3)</td>
<td>9.37±15.95</td>
<td>7.91±9.53</td>
</tr>
<tr>
<td>Type of invasion pattern</td>
<td>Degree 1 (n=3)</td>
<td>64.32±32.21</td>
<td>13.51±6.73</td>
</tr>
<tr>
<td></td>
<td>Degree 2 (n=4)</td>
<td>34.27±31.47</td>
<td>17.67±17.79</td>
</tr>
<tr>
<td></td>
<td>Degree 3 (n=5)</td>
<td>41.37±33.52</td>
<td>19.32±27.63</td>
</tr>
<tr>
<td></td>
<td>Degree 4 (n=3)</td>
<td>30.67±37.42</td>
<td>22.46±25.75</td>
</tr>
</tbody>
</table>

According to the data from Table 1, the membrane E-cadherin reactivity decreased in parallel with decreasing of tumor differentiation degree and with the increase of invasion pattern degree. Thus, the E-cadherin membrane pattern was noticed in well-differentiated form of tongue OSCC with type 1 of invasion pattern, being more prevalent in the center of tumor islands (Figure 1, A and B). The E-cadherin cytoplasmatic pattern was more obvious in poor differentiated tongue OSCC variants with type 4 of invasion pattern, this immunoreactivity being more prevalent at the periphery of neoplastic proliferations and at the advancing edge (Figure 1, C and D).

### Vimentin reactivity

The reaction was present in all the investigated cases with cytoplasmatic reactivity being more obvious at stromal level. Strictly, at the carcinomatous proliferations the vimentin reactivity was present in 11 cases. The semi-quantitative reactivity assessment of these cases revealed the prevalence of score 1 in nine cases and respective those tumors that were only membrane stained and respective those tumors that present only cytoplasmic reaction and then we tried to correlate these scores with the main morphological parameters (TNM stage, tumor differentiation degree and type of invasion pattern) (Table 2).

<table>
<thead>
<tr>
<th>Morphological parameter</th>
<th>Subcategories</th>
<th>Membrane E-cadherin reactivity (mean ± SD)</th>
<th>Cytoplasmic E-cadherin reactivity (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNM stage</td>
<td>I (n=5)</td>
<td>41.23±36.32</td>
<td>15.43±15.21</td>
</tr>
<tr>
<td></td>
<td>II (n=3)</td>
<td>39.45±28.31</td>
<td>17.23±16.57</td>
</tr>
<tr>
<td></td>
<td>III (n=4)</td>
<td>20.34±23.52</td>
<td>18.73±24.76</td>
</tr>
<tr>
<td>Differentiation degree</td>
<td>Well (n=4)</td>
<td>55.73±31.53</td>
<td>25.37±21.63</td>
</tr>
<tr>
<td></td>
<td>Moderate (n=8)</td>
<td>37.43±29.12</td>
<td>16.31±18.72</td>
</tr>
<tr>
<td></td>
<td>Poor (n=3)</td>
<td>9.37±15.95</td>
<td>7.91±9.53</td>
</tr>
<tr>
<td>Type of invasion pattern</td>
<td>Degree 1 (n=3)</td>
<td>64.32±32.21</td>
<td>13.51±6.73</td>
</tr>
<tr>
<td></td>
<td>Degree 2 (n=4)</td>
<td>34.27±31.47</td>
<td>17.67±17.79</td>
</tr>
<tr>
<td></td>
<td>Degree 3 (n=5)</td>
<td>41.37±33.52</td>
<td>19.32±27.63</td>
</tr>
<tr>
<td></td>
<td>Degree 4 (n=3)</td>
<td>30.67±37.42</td>
<td>22.46±25.75</td>
</tr>
</tbody>
</table>

In the following table are presented the semi-quantitative scores of carcinomatous cells to vimentin according to the main morphological parameters (Table 2).

In addition, we noticed higher vimentin reactivity at the advancing edge compared with the superficial part of tumors, the reaction being more obvious in type 3/4 of invasion pattern (Figure 2, A and B). Regarding the tumor differentiation degree, vimentin reactivity was
higher in poor differentiated tumors (Figure 2C). Data analysis revealed that vimentin parenchyma reactivity seemed also to vary with TNM stage, the highest immunoreactive tumor cells percentage being recorded in more advanced stages (III–IV).

Statistically, the E-cadherin and vimentin reactivity were inversely correlated, respective well-differentiated tumors aggregated with low invasion pattern, and earlier stages had higher E-cadherin staining and low vimentin reactivity. In addition, while E-cadherin reactivity was more obvious in the center of tumor proliferations and in the superficial part of tumors for vimentin the reactivity prevailed at the periphery of tumor proliferations and at the advancing edge.

Figure 1 – (A) Tongue oral squamous cell carcinoma (OSCC) – well-differentiated tumor, E-cadherin membrane positive reaction in carcinomatous cells from centre of tumor islands. IHC for E-cadherin (brown)/vimentin (red), ×40. (B) Tongue OSCC – well-differentiated tumor, type 1 of invasion pattern, E-cadherin membrane positive reaction in carcinomatous cells from centre of tumor islands. IHC for E-cadherin (brown)/vimentin (red), ×40. (C) Tongue OSCC – moderate differentiated tumor, E-cadherin cytoplasm positive reaction in carcinomatous cells. IHC for E-cadherin (brown)/vimentin (red), ×40. (D) Tongue OSCC – moderate differentiated tumor, type 3 of invasion pattern, E-cadherin cytoplasm positive reaction in carcinomatous cells. IHC for E-cadherin (brown)/vimentin (red), ×100. (E) Tongue OSCC – moderate differentiated tumor, vimentin reactivity in carcinomatous cells from the invasion front, type 2 of invasion pattern. IHC for E-cadherin (brown)/vimentin (red), ×100. (F) Tongue OSCC – moderate differentiated tumor, acantholytic area, intense vimentin reactivity in acantholytic carcinomatous cells. IHC for E-cadherin (brown)/vimentin (red), ×100.
isolated tumor cells (Figure 2E).

...when this presented as small carcinomatous nests or as reactivity was noticed at the invasion front, especially tumor periphery (Figure 2D). Overall, N-cadherin tumor most reactive carcinomatous cells being those from the invasion pattern and in advanced tumor stages (all five investigated cases), in tumors with type 3 of was more obvious in poor differentiated tumors (in all three investigated cases), with type 4 of invasion pattern (Table 3).

Discussion

The EMT process is characterized by a range of cellular and morphological changes including loss of cell polarity and intercellular adhesions, increasing motility, and acquisition of a mesenchymal phenotype [11, 12]. Such phenotypic changes are accompanied by alterations at the molecular level including suppression of E-cadherin gene expression.

Reactivity to E-cadherin

E-cadherin is a major intercellular adhesion molecule and plays a crucial role in development, and maintenance of epithelial cell polarity and tissue architecture [13]. Loss of E-cadherin expression was associated with increased invasiveness, metastasis and a poor prognosis for tongue squamous cell carcinoma [14, 15].

In our study, although we noticed E-cadherin reactivity in all the investigated cases, semiquantitatively the maximum reactivity was recorded in only two cases, most of them having a score of 2 (73.3%). The pattern of reactivity was dual at membrane and cytoplasmic level with the membrane pattern decreasing simultaneously with the decrease of the differentiation degree and with the increase of invasion pattern type, while the cytoplasmic pattern was more obvious in the poorly differentiated tumor types, with type 4 of invasion pattern, predominantly at the periphery of carcinomatous proliferations and at the advancing edge.

Some studies have shown that E-cadherin expression in OSCC is associated with higher incidence of undifferentiated forms and a higher frequency of lymphatic metastasis [16–18]. However, other authors have shown that they are not significant differences between E-cadherin expression pattern and its semiquantitative score among patients with and without lymphatic metastasis [19–22].

Also, some authors have shown a decrease in E-cadherin expression in squamous cell carcinomas with high rate of malignancy, which could be explained by the relationship between the degree of epithelial differentiation and expression of intercellular adhesion mole-
The immunohistochemical investigations of cadherin "switch" during epithelial-mesenchymal transition...

Contrary to these, other authors could not establish an association between E-cadherin expression and the histological grade of malignancy in squamous carcinomas from various sites [20, 24, 25]. Responsible for these contradictory results seems to be: the size of the investigated tumor specimens, using of various malignancy grading systems and the tumor cellular heterogeneity [26]. In addition, a number of studies have shown that in patients with head and neck squamous cell cancer that presented a low rate of E-cadherin expression has been recorded a significant decrease in the rate of survival [25, 27].

**Figure 2** – (A) Tongue oral squamous cell carcinoma (OSCC) – well-differentiated tumor, tumor superficial part, reduced vimentin reactivity in carcinomatous cells. IHC for E-cadherin (brown)/vimentin (red), ×100. (B) Tongue OSCC moderate differentiated tumor, type 3 of invasion pattern, increased vimentin reactivity of carcinomatous cells. IHC for E-cadherin (brown)/vimentin (red), ×40. (C) Tongue OSCC – poor differentiated tumor, type 3 of invasion pattern, increased vimentin reactivity of carcinomatous cells. IHC for E-cadherin (brown)/vimentin (red), ×40. (D) Tongue OSCC – moderate differentiated tumor, prevalent cytoplasmic N-cadherin reactivity of carcinomatous cells that cytoplasmic coexpressed E-cadherin. IHC for N-cadherin (brown)/E-cadherin (red), ×100. (E) Tongue OSCC – poor differentiated tumor, cytoplasmic N-cadherin reactivity restricted to few nests of carcinomatous cells. IHC for N-cadherin (brown)/E-cadherin (red), ×100. (F) Tongue OSCC – moderate differentiated tumor, cytoplasmic coexpression of N-cadherin/E-cadherin in carcinomatous cells. IHC for N-cadherin (brown)/E-cadherin (red), ×100.
Reactivity to vimentin

Vimentin as a major constituent of the large family of intermediate filaments is typically expressed in normal mesenchymal cells and plays a major role in maintaining cellular integrity and its resistance against various stresses [28, 29]. This protein has gained great importance as an usual EMT marker, a process of cell reprogramming by which epithelial cells acquire a mesenchymal phenotype forcing them to change their morphology and to become mobile [9]. Moreover, cells expressing vimentin have a higher mobility, being capable of invasion and proliferation, and also have tumorigenic potential [30]. Also, an increased vimentin expression was detected in epithelial cancers diagnosed as being poorly differentiated or as variants of sarcomatoid carcinoma, and also in high invasive patterns [31].

Many studies have detected an increased expression of vimentin in OSCCs or in squamous carcinoma cell lines [14, 31–35], and it overexpression was associated with local recurrence, invasiveness and metastasis [14, 31, 34]. Chaw et al. (2012) have observed a significant increase in vimentin expression from normal oral epithelium to dysplastic epithelium and respective to OSCC [36]. The authors concluded that the reduction of E-cadherin expression and vimentin overexpression in dysplastic and neoplastic tissues would suggests that the EMT process may occur and would play a key role in the development of OSCC. In addition, it was proved a vimentin overexpression in the invasion front, which would be in line with the role played by vimentin in cell migration, and may explains the already proven correlation between vimentin expression and invasive behavior of these tumors [31]. Moreover, it is known that cells from the invasion font are more aggressive having a higher potential for metastasis [37]. At the basis of such behavior in fact lies the EMT process, fact that was confirmed by expression at this level of a series of genes involved in this process, including vimentin [14].

In fact, it is known that primary head and neck squamous tumors that had a reduced E-cadherin expression together with vimentin overexpression also had a higher incidence of distant metastasis [34]. Thus, it has been argued for E-cadherin and vimentin usefulness as dual predictive biomarkers for metastasis in head and neck squamous cell carcinomas.

We proved parenchyma tumor reactivity for vimentin in only 73.3% of investigated cases with only 13.33 of these cases that had more than 10% immunoreactive tumor cells to this marker. The reactivity was more obvious in tumor cells from the periphery of proliferative islands, but also in acantholytic carcinomatous cells with oval or fusiform morphology from the centre of neoplastic islands. However, the semiquantitative score of vimentin reactivity correlated with the degree of differentiation, clinical stage and pattern of invasion, its immunoreactivity being obvious in poorly differentiated tumors, in those with high-grade invasive pattern and in advanced stages of the disease.

Investigation of E-cadherin/vimentin coexpression showed that there is a direct inverse correlation, namely a decrease of the E-cadherin reactivity simultaneously with an increase of vimentin reactivity along with a decrease in the degree of differentiation, an increase of the invasion pattern and respective with the increase of clinical stage. This was evident especially at the advancing edge of the investigated tongue squamous carcinomas.

Reactivity to N-cadherin

Unlike E-cadherin, N-cadherin is normally expressed in tissues derived from the mesoderm and neuroectoderm and not by normal squamous epithelium [38, 39]. Also, if E-cadherin plays important roles in maintaining of epithelial phenotype and in inhibition of cell growth dependent on cell density, N-cadherin has somewhat contrary effects being associated with mesenchymal cell phenotype and increasing their motility and invasion [40].

Many previous studies have demonstrated a functional relationship between the expression of E-cadherin and other non-epithelial cadherins, namely N-cadherin [41]. Specifically, it appears that N-cadherin overexpression would decrease the E-cadherin expression, a phenomenon called E-cadherin/N-cadherin “switching” (EN-switch), and consecutively the epithelial cells expressing N-cadherin would have an increased motility and invasiveness [42].

In addition, the E-cadherin replacement in these cell types does not seem to affect the aggressive phenotype [43, 44]. This EN-switch and aberrant expression of N-cadherin that plays major roles in the EMT process represents an independent prognostic factor in cancer progression, fact that was confirmed in gastric carcinomas, prostate and oral squamous cell carcinoma [14, 45, 46]. On the other hand, other studies have shown that EN-switch is required to increase the motility but not to induce morphological changes that accompany the EMT process [47].

In head and neck squamous cell carcinoma, the N-cadherin expression and its involvement in tumor progression remains a controversial topic [48–50]. Previous studies reported in OSCCs a rate of N-cadherin expression ranging from 37 to 52.4%, and the belief that EN-switch is involved in progression of these carcinomas [25, 50, 51]. Hashimoto et al. (2012) observed that the N-cadherin expression was limited in OSCCs and with a lack of correlation with clinicopathological parameters and the reduction of E-cadherin expression [52]. Di Domenico et al. (2011) noted a nuclear reactivity for N-cadherin in OSCCs, especially in dedifferentiated variants that also were characterized by a poor prognosis [48]. The same authors have shown the existence of significant correlations between N-cadherin expression and the differentiation degree and disease stage. In addition, cases that presented N-cadherin overexpression (>61% cases) were characterized by a poor prognosis and had a greater tendency to relapse and/or metastasis. Also, Li et al. (2009) has shown that overexpression of N-cadherin and beta-catenin in association with loss of E-cadherin expression plays an important role in invasion and metastasis of tongue squamous cell carcinoma [51]. However, the authors could not establish correlations between the expression/subexpression of these EMT markers and other morphoclinical parameters (gender, lymphatic status and clinical stage). The authors concluded that in particular N-cadherin may be an effective molecular therapeutic target in the treatment of OSCCs.
to only 33.33% of the investigated cases, the recorded semiquantitatively score was score 1 (namely less than 10% of tumor cells were positive). This immunoreactivity was prevalent in poorly differentiated forms, in tumors with type 3 invasion pattern and in advanced disease stages (stages III–IV). However, given the limited number of positive cases and the low number of reactive tumor cells we could not establish significant correlations between its expression and the other investigated morphoclinical parameters. Coexpression with E-cadherin was rarely observed and only in few proliferative islands from the advancing edge, the E-cadherin being membrane expressed, and the N-cadherin in cytoplasm. In fact, the expression patterns of these two cadherins were opposed to each other. Thus for N-cadherin the reactivity was predominantly cytoplasmic and particularly present at the periphery of the proliferative islands from the advancing edge, especially in the poorly differentiated forms of investigated tongue squamous cell carcinomas.

### Conclusions

Investigating the E-cadherin/vimentin coexpression in tongue squamous cell carcinomas, we found that at the advancing edge the E-cadherin reactivity decreased while vimentin expression increased and this EMT phenotype was correlated with the decrease of differentiation degree, with the increase of the type of invasion pattern and with increasing stages of the disease. In addition, the N-cadherin/E-cadherin coexpression was rarely noticed and only in few proliferative islands from the advancing edge, especially in poorly differentiated tongue squamous cell carcinomas with E-cadherin mainly membrane expressed while N-cadherin was preferential expressed in cytoplasm. In conclusion, these EMT markers could be used for prognostic stratification of patients with tongue squamous cell carcinomas in order to establish a personalized therapy.

**Author contribution**

All authors have contributed equally to the present work.

### References


Corresponding author
Claudiu Mărgăritescu, Professor, MD, PhD, Department of Pathology, Faculty of Dentistry, University of Medicine and Pharmacy of Craiova, 2 Petru Rareș Street, 200349 Craiova, Romania; Phone +40740–152 550, e-mail: c_margaritescu2000@yahoo.com

Received: March 28, 2014     Accepted: November 7, 2014