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MIGHT THE DETECTION OF ALTERATIONS IN E-CADHERIN EXPRESSION IMPROVE THE TREATMENT OF SPORADIC GASTRIC CANCER?

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Abstract: *The risk of GC incidence and mortality has declined in most country, but an acceptable overall five-years survival (52%) has been reached only in Japan, due to the mass screening that has lead to early discovery. A reliable prognostic marker, could guide the therapeutic strategy, to provide a molecular target for therapies and act as a classic tumor marker in follow up, is strongly felt. E-cadherins (E-cad) could be such marker.*

E-cad is an adhesine, expressed in the adherent junction of epithelial cells. The mutation or deletion of the E-cad gene (CDH1) has been found in some familial cancers but is common even in many sporadic cancers and is associated with poorly differentiated histotypes and poor prognosis. Many studies have indagated its reliability as tissutal and serum marker but the large variety of methods of evaluation of E-cad expression makes difficult to compare the results.

Nevertheless the role of E-cad appears very interesting.

Once excluded the specific issue related with familial GC, where in the presence of suspected familial GC, after an anamnestic careful screening, CDH1 test may identify the subset of patients in whom an early prophylactic total gastrectomy could reverse a fatal destiny, E-cad shows a particular clinical interest in three principal setting:

1) *In early GC, on biopsy sample, together with other imaging technique, might identify the cases that, although apparently favourable, could benefit from a more aggressive lymphadenectomy.*
2) *As soluble marker might be of help in follow up, providing a reliable clue for early diagnosis of recurrence. Thus further studies are needed to validate the efficacy of this marker.*
3) *In the future, a targeted therapy could achieve the results that are still now forbidden to surgery because of a late diagnosis.*

INTRODUCTION

Gastric cancer (GC) is still the fourth for frequency among solid neoplasms and the second leading cause of cancer death, with respectively over 934,000 new cases and 720,000 deaths per year. Its demography has changed in last decades but the large geographical differences in distribution are still and even more strong. The risk of GC incidence and mortality has declined in most country, due to improvements in preservation and storage of foods and reduced *Helicobacter pylori* transmission in childhood[1].

Nevertheless East Asia (China, Korea, Japan), Eastern Europe and some areas of Central and South America are still high risk countries. China alone is burdened by 42% of cases of GC. An acceptable overall five-years survival (52%) has been reached only in Japan, due to the mass screening conducted by the Government since 1960, that has lead to early discovery[2]. Early gastric cancer (EGC), defined as an adenocarcinoma restricted to the gastric mucosa or submucosa, irrespective of the presence or absence of lymph node metastases, is the key of such success. Five-year survival rate for EGC exceeds 90%, depending on the degree of

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tumor invasion (mucosa type T1a or submucosa type T1b) and the presence of metastatic lymph nodes. But when the tumor has gained the *muscularis propria* or serosa the 5-year survival rate lowers to10%-20% at most[1].

In most western country, where the risk is low, the mass screening is felt as not cost-effective. Though, having to face with a heterogeneous and still fatal disease, the need for a reliable prognostic marker, useful both to guide the therapeutic strategy, to provide a molecular target for therapies and to act as a classic tumor marker in follow up, is strongly felt. E-cadherins (E-cad) could be such marker[3].

Could the identification of altered expression of E-cad in biopsy sampling or in blood play a role in definition of therapeutic strategy, from the size of resection to the choice of lymphadenectomy to be performed? Or even help the oncologist in choosing the chemotherapy or the immune therapy more fit to the target? The present article would answer to these questions.

E-CADHERINE

One of the principal characters of a cancer cell is the loss of adhesion that makes the cell able to leave its place and invade adjacent tissues and metastasize, an event usually considered a late step in tumorigenesis. The adhesion is determined by the expressione of cytoplasmic proteins: the adhesines. Among them the cadherins, that act by linking with other cytoplasmic proteins, i.e. cathenins, are actually well known. They can be classified in 4 principal group on the basis of their tissutal distribution, E-cadherin (epithelial), P-cadherin (placental), N-cadherin (neural) and L-CAM (liver)[4].

E-cad is expressed in the adherent junction of epithelial cells. The reduced or loss of expression of this protein leads to the disruption of epithelial lines and is considered a fundamental step in invasion and metastasization. The mutation or deletion of the E-cad gene (CDH1) has been found in some familial cancers but is common even in many sporadic cancers. Both in familial and sporadic GCs, CDH1 is associated with poorly differentiated histotypes and poor prognosis[4].

The loss of expression, leading to a loss of adhesion, can be due to different mechanism: a) promoter hypermetilation, which acts by antagonizing tumor suppressors[5]. This mechanism can be related to infection[6]; b) activation of SNAIL[7] and SLUG[8], transcriptional repressors of E-cad; c) germline or somatic alteration of CDH1, the gene that encodes E-cad[1].

Mutations of this gene have been recognized in many neoplasms but in GC they have a prominent role[1].

THE MEASUREMENT OF EXPRESSION AND THE CUT OFF VALUES

The study of expression of E-cad has been performed usually by Immunohistochemical assays (ICH), namely by direct staining of hystologic sample with commercial mouse monoclonal antibodies[9] but the definition of "reduced" is extremely various.

Aoki considered normal a staining in cancer tissue of 80% or more respect to normal tissue from the same patient. Under this value the expression was defined "reduced"[10].

Begnami et al[11] considered positive the staining if was present in >10% of the neoplastic cells.

In other studies[12,13] E-cad expression was evaluated by defining normal the expression in cancer cells that stained like the normal cell of the same patient, while those which stained more weakly or not at all were defined as having "reduced" expression.

Blok[4] used a score system taking in account the percentage of cells expressing E-cad and the intensity, with the expression of normal tissue as reference. The score was calculated as follows: positive cells < 10% = 0, 10-30% = 1, 30- E-cad 60%= 2, > 60% = 3; staining intensity was semi-quantitatively expressed as percentage of reference intensity. The product of the two scores was the final score. Four sections from each tumour were scored to correct the value for tumour heterogeneity.

Stanculescu used a similar scoring system that combined a qualitative score (0, 1+, 2+, 3+ from absence of staining to strong reaction expressed by intense brown colour) with a quantitative evaluation of percentage of cells (0 = negative, 1 = < 25%, 2 = 25-50%, 3 = 50-75%. The value obtained by multiplying these scores[14] was classified as 0 = negative, 1-4 = very low or +, 5-8 = low or ++, 9-12 +++.

Chu used a similar score with different notation: no staining in fewer than 10% of tumor cells; +, weak staining in only 10%-50% of tumor cells; ++, moderate staining in 50%-75% of tumor cells; and +++, strong staining of more than 75% of tumor cells[15]. Similar the score proposed by Jawhari: 0, no staining; 1, cytoplasmic staining; 2, cytoplasmic and membranous staining in the same case; 3, normal membranous immunoexpression; abnormal patterns: scores 0, 1 and 2; normal pattern: score 3[16].

Almeida[17] slightly modified the interpretation of Jawhari scores pointing out the absence of membranous expression scores (0 and 1) versus the presence of membranous expression (scores 2 and 3). Chen[9] used a quantitative percentage score.

Li prefers the simple localization of E-cad: membranous when only localized in the cell membrane and abnormal if lacking or expressed in the cytoplasm[18].

It is evident that such discrepancies can lead only to not homogenous results. Xing et al, in their metanalysis tried to put an order in these results grouping the articles selected in two main groups, based on the value of cut-off, less or more than 50[19].

E-CADHERIN AND HISTOTYPE

Gastric cancer is not an homogeneous disease. Indeed, following Lauren classification GC can be classified as diffuse type or intestinal type, the first having a lower incidence and an higher aggressive with earlier nodal and distant metastases. The intestinal type, related to *Helycobacter pylori* infection, is that which incidence has lowered in western countries. Thus, on an epidemiologic basis can be distinguished a sporadic GC (90% of cases) and a familial GC (10%). This last is more prone to present the diffuse type, leading in 1999 to the description of Hereditary Diffuse GC-HDGC. HDGC nevertheless represents only a small percentage of cases of Familial GC, about 1 to 3%[20]. In 45% of these cases a mutation in CDH1 has been recognized. Actually, thanks to this discovery, early total gastrectomy is a clearly defined tool in prevention of GC in selected groups of patients[21]. Familial Intestinal GC however failed to show significant association with germ line mutations of CDH1[22].

Even when a germ line mutations of CDH1 cannot be demonstrated, a somatic alteration can be observed in many GC, structural or epigenetic, and can be recognized both in diffuse and intestinal GC, either in sporadic or in familial setting[12]. Even in these cases they are usually associated with a poorer prognosis[22].

The true reliability of E-cad expression is nevertheless still not clear because the comparison of the results is very difficult.

Expression of E-cad is usually high in non tumor gastric tissue (85%-100%)[9,18] while it is defective or lost in 17-92% of GC[13,15,18,23].

Most of studies agree on being E-cad loss of expression more frequent in diffuse hystotipe (65%-90%), less in mixed (31%), more infrequent in intestinal type (20%)[9]. Some other found a still significant but less important difference (89-90-67.9% respectively)[24] or 60 *vs* 45% respectively in intestinal and diffuse type[14].

EARLY GASTRIC CANCER

In Early GC the need for a reliable prognostic marker, highly predictive for nodal metastases is deeply felt, because small superficial cancer are actually candidate to conservative endoscopic resection or laparoscopic wedge resection without lymphadenectomy. The identification in biopsy samples of such marker could be of great value in the selection of the therapeutic approach.

So the expression of E-cad has been compared with the usual clinico-pathological features and with nodal status in several studies, leading to interesting results.

Blok et al in 1999, in a study on 45 samples from early GC, found an inverse correlation between E-cad expression and grade of differentiation in intestinal-type carcinomas with median product scores of 225, 100, and 50 for highly, moderately and poorly differentiated carcinomas, respectively ($p < 0.0001$). In some early carcinomas with prevalent intestinal hystotype and smaller areas with a diffuse growth pattern, these last presented a significant reduction in E-cad expression. In this paper was evident a relationship with Lauren classification, though the Authors preferred to refer the low levels of E-cad expression to the lower differentiation than to the histotype. Thus, a not significant difference was found as regard the nodal status, being the expression of E-cad absent or reduced in 80% of node positive and in 60% of node negative. Also the overall survival was independent from E-cad status[4].

Similar results had been presented by Joo et al[25] in 2002. They studied the expression of E-Cad and catenins on a group of 108 EGC finding a decreased expression of E-cad in 43.5% of cases. Reduced expression of E-cad and catenins correlated with poorly differentiated and diffuse-type cancers, but not with the patient's age, gender, tumor size, location, macroscopic type, depth of invasion, or lymph node metastasis.

Cai et al[26] in 2001, from a group of 162 early GC, tested 135 of them for E-cad expression. In 77 (57%) they found a reduced expression of E-cad. The loss was strongly related with the tumor size being only present in 2/14 (14%) of those < 1cm in diameter, in 16/33 (48%) of those 1.1-2 cm and in 59/88 (67%) of >2 cm. A strong relationship with involvement of the submucosa (m 48%, sm 1 54%, sm 2 88%) and with lymphatic (no 54% vs 92%) or vascular invasion. (no 48% vs 30%). A similar strong relationship was found with the presence and the type of nodal metastases, classified as number of nodes (No, 1, 2-3 and >3, respectively), features (no, single or scattered cell or clusters) and location (No, Group 1, Group 2 or 3). They concluded that E-cad immunostaining on biopsy sample could be helpful, together with endoscopic relief, in defining the limits of excision, because small (<1 cm) m-cancer could undergo to endoscopical mucosal resection or to laparoscopical wedge resection, being nodal involvement extremely rare. For m-cancer 1-2 cm a

D1 lymphadenectomy could be sufficient, while in m-cancer >2 cm or m-cancer a radical D-2 lymphadenectomy is mandatory.

The study by Aoki et al[14] was in agreement with this view. They examined 107 submucosal, early GC, classified following Nakamura as gastric type, intestinal type, mixed and Lack of Mucin (LOM), have been examinated finding that the expression of E-cad was similar in the four groups. Nodal metastases were found in eleven cases, with an higher, but not significant, rate in papillary GC. The lymph node metastasis rate (46.2%) was significantly higher in gastric-phenotype carcinomas with reduced E-Cad expression than in carcinomas of other phenotypes ($p < 0.05$). In the light of a possible endoscopic resection for apparently favourable GC, a molecular and phenotypic study of the lesion before surgery could drive the treatment in the sense of a more traditional and radical approach[10].

A similar conclusion is found in the study by Kim et al[32] and we share their view: "Therefore, even if the tumor stage is low and no histological lymph node metastasis is found, extensive lymphadenectomy and micrometastatic research should be performed in the patient who is preoperatively found to have reduced E-cad expression".

More recently, even Lee et al[28] evaluated E-cad expression as a possible marker for lymph node metastasis of early GC. Wanting to take in account the tumor heterogeneity they assessed the protein expression in mucosa and submucosa in different tumors: 37 pN1-3 early GCs and depth- and size-matched 31 pN0 EGCs were compared with 72 early GCs including 14 pN1-3 EGCs as test set. Protein expression for beta-catenin, E-Cad, N-cadherin, galectin-3, c-MET, TrkB, and Ki-67 was assessed by immunohistochemistry in mucosal (-m) and submucosal (-sm) portions of tumor. The multivariate analysis showed that altered expression of E-cad in submucosa, tumor size larger than 3 cm, diffuse type histology, and lymphatic invasion were predictive of nodal invasion with 100% sensitivity and 90.9% specificity in 21 submucosal early GC. Their results were suggestive of being the proliferative activity of tumor in submucosa an independent predictor for LN metastasis in early GC.

This date is surely interesting but, in our opinion, being based on the analysis of the specimen in its totality, is not so useful as the simple level of E-Cad in the bioptic sample.

E-CADHERIN AND LYMPH NODE STATUS

Even apart from EGCs, the relationship between the expression of E-cad and stage, metastases and survival is investigated last decades of 20th century[29,30].

Nevertheless, the relationship between loss of E-cad expression and Lymph node status is far from well defined.

In the study by Chen et al[9] among 84 patients with GC, a loss of E-cad expression was present in 36% of 58 Node positive patients and in 30% of N0, slightly lower but not significant.

Stanculescu et al found a relationship between alterations in expression of E-cad and nodal status only under the profile of number of nodes involved, being lower the values of E-cad in cases with higher number of metastatic nodes[14].

EXPRESSION OF E-CADHERIN AND SURVIVAL

Some Author presented data supporting a quite striking difference in survival in favour of GC expressing high levels of E-cad. Chu et al reported that the patients with high E-cad expression level had a 5-year-survival rate of 93.59%, significantly different from those with low E-cad expression in whom the rate was just of 12.8%. Nevertheless, the same Author concludes that there aren't data supporting that E-cad might be considered as an independent prognostic factor[9].

A recent metanalysis by Xing et al[14] has verified the presence of a relationship between expression of E-cad, measured by IHC, clinico-payhologic features and prognosis. The study collected data from 4383 patients grouped in 26 studies of various size, from 30 to 564, and various geographical origin. Cut off values were heterogeneous and were generically grouped in 50 or <50. The conclusion of the studies were quite different: in 15 studies the reduced expression of E-cad turned out to be a poor prognostic factors, while in 10 it was not and in the last was associated to a surprising better prognosis. The metanalysis though reported an effective relationship between decreased expression of E-cad and poor prognosis. These results weren't affected by geographical origin, sample size or year of publication, while a considerable heterogeneity was introduced by cut-off value.

The more significant relationship was presented by Asian studies and by those in which cut off value was higher. The clinico-pathological evaluation resulted in defining the E-cad status as being an independent prognostic factor, as TNM stage, depth of invasion, nodal status, distant metastases, and Grading. The Author concludes that decreased E-cad might be a predictive factor of lymph-node and distant metastases and of poor Overall Survival (OS). In our opinion another interesting clue that can be inferred from their analysis, by a clinical point of view, is the need for a clear definition of cut off

value, that in the studies examined ranged between 5 and 90%, leading to heterogeneous and not comparable results[9,7,14,16,27,31,32].

A paper by Anzai et al[5] examined 25 sample of GC from patients followed for at least 4.5 years with the scope of elucidate the correlation between stage and expression of a large panel of molecular markers including E-cad. Since the values for each marker were clearly indicated, case by case, we re-examined their results looking for a relationship between E-cad expression, stage at diagnosis and survival. We found that the median value of E-cad expression was significantly related to stage: IV stage presented with mean values of 39.75 ± 9.125 and all the patients died after liver or peritoneal recurrence in maximum 13 months, median 6 months; a significant difference was seen within III stage, between "with recurrence" and "not recurrence". They showed mean values of E-cad respectively 44 and 61. The III stage with no recurrence showed the highest expression even respect to II stage (51), while the mean follow up was similar, 60 months vs 58.

Zhang et al[33] found the expression of E-cad aberrant gene in 47.2% on 180 GC samples, a rate significantly higher than in normal tissues. So were even the gen Wnt-1 and Beta-catenin. Thus, these indicators were significantly related to tumor size, tumor invasive depth, lymph node metastasis, pTNM stage, differentiation and five-year survival rate. Similar results were obtained By Zhong et al[34] in a study on 118 cases.

Uchikado et al[12] evaluated the predictive value of E-cad and SLUG in 164 GC patients. The 5-year survival rate of patients with tumors with normal E-cad expression was 88.6%, vs 63,5% for patients with reduced E-cad expression with a significative difference ($p = 0.022$).

Begnami et al studied the relationship between a signature, made of 28 antibodies, including those for E-cad, and clinico-pathologic features in 428 GC. Even if the study was aimed to check the validity of such panel the results were statistically examined so as to elucidate the significance of each single determination. E-cad immunostaining was significatively associated with intestinal type. The study did not revealed any specific relationship with survival although inferring a prospective possible utility of the signature in the selection of target therapies[11].

In the study by Li (2012) E-cad was expressed in 82.4% (94/114) of GC tissues but only in 45 (39.4%) samples, E-cad showed the normal pattern, with localization in the sole cell membrane. The remaining 69 showed an abnormal pattern with lacking or cytoplasmic expression of E-cad. The abnormal expression was associated with the tumor depth ($p = 0.003$), lymph node metastasis ($p = 0.001$), distant metastasis of GC ($p = 0.001$) and advanced clinical stage ($p = 0.001$). OS well correlated with E-cad expression with a mean survival of 61 months for patient with membranous expression and 27,881 months for those with abnormal expression (p-value < 0.001). Nevertheless, though the prognostic value of E-cad is once more confirmed, it can be considered of help for surgeon in defining treatment strategy[18].

Sereno et al studied a panel of possible predictive of relapse factors on 44 samples from GC patients undergone surgery and adjuvant chemotherapy, finding E-cad alterations positively related with recurrence. In fact, the expression of E-cad was conserved only in 5/15 (33.3%) cases with recurrence and in 19/29 (65%) of the no recurrence cases. The E-cad expression was thus related to the other molecular and clinicopathologic features[35].

The study by Corso et al[22], aimed to correlate the somatic expression of CDH1 with survival in 246 patients without germline alterations. The study included both familial and sporadic GC, either intestinal or diffuse, and distinguishes the cases on the basis of the type of somatic alteration, epigenetic (promoter Hypermetilation alone) or structural (Loss of Hetrozigosity). In this study the distribution of alterations was not statistically different between intestinal or diffuse type. Along with expected results, confirming the poor survival in carriers of CDH1 structural alterations, was observed surprisingly that the worst prognosis belonged to Intestinal type with these alterations, and among them to FIGC, although FIGCs are usually considered at better prognosis than diffuse type. Epigenetic alterations were instead associated with a better prognosis in all settings. This evaluation has been demonstrated efficient in identifying subset of GC that, although showing an intestinal histotype presented a poor prognosis. So the early identification of CDH1 alteration in biopsy sample could be useful in selecting the patients for whom an extended lymphadenectomy (D2 or more) would be more appropriate[22].

In this study the immunostaining for altered E-cad expression did not correlate with alterations of CDH1 because about 68% of sample positive did not show CDH1 alterations, so suggesting alternative mechanisms by which E-cad could decrease or be lost, such as miR-101 down regulation. Nevertheless, even in this study, E-cad presented with aberrant expression in 73.4% of GC and in 14.5% was completely loss. Moreover, in both familial and sporadic setting, the complete loss of E-cad expression was significantly associated with diffuse histotype[22].

SOLUBLE E-CADHERIN AS A SERIC TUMOR-MARKER

Since early '90s E-cad have been studied as a possible tumor marker since its elevation had been shown in up to 57% of GC patients[36,37]. The presence of these low weight protein can be explained by the disruption of the original protein by proteases of neoplastic origin, so that the protein can pass in the blood stream and be measured. In healthy individuals the usual level of E-cad has been found 2 μg/ml[36].

The method of test is usually ELISA.

Chan et al[37] used immunostaining with colorimetric automatic measurement respect to standard values, then arbitrarily defined significant an increase in E-cad value of ≥50% respect preoperative values, at any time during the follow up. The study led to define a cut-off value of 10.000 ng/mL. Similar cut off have been set in other studies[38]. Chan measured sE-cad and CEA in the serum of 69 patients before and after curative surgery up to 60 months (median 36) so to evaluate the reliability of E-cad as early marker of recurrence. sE-cad showed a sensibility of 59% and a specificity of 75%, within the six months immediately after surgery. Thus, a rising in its levels preceded the diagnosis in all patients with recurrence. The median anticipation was of 13 months (range 3-20 months). Compared with behaviour of CEA levels (sensitivity 6% at 3 months post surgery and 6% at 6 months post surgery; $p = 0.004$ and $p < 0.0001$, and diagnostic anticipation 4 months range, 1-20 months-respectively), sE-cad performed better and earlier.

In 1998 Gofuku et al[39] presented his study on 81 patients with GC. Sixty-seven of them had abnormally high level of sE-cad in the serum, while CEA was increased in just 4.4% of cases. Grouping the patients by expression of E-cad, those with abnormal but not lost expression, had the higher levels of sE-cad.

Tsalidikis et al. confirmed this result, with a slight, not significant, difference in the setting of optimal cut off that in their study was 9.9 microg/mL. They found a sensitivity of 72.7%, specificity of 80.8% and global accuracy of 76.3%. Setting an higher cut off value (17.60 microg/mL) the global accuracy remained similar but lowered the sensitivity and raised the specificity (respectively 75%, 57%, 83%). Thus, survival was poorer in patients with increased sE-cad (median, 7 months vs 39 months, $p = 0.0002$)[38].

The argument remains nevertheless controversial. Juhasz et al[40] gave particular importance to the rising of sE-cad in advanced intestinal type cancer, while other AA did not find any significant elevation of sE-cad in serum of GC patients[41]. Another very interesting study, conducted by Pedrazzani et al[42], even not aimed to early diagnosis of recurrence but to find a relationship with survival, showed an influence of age on levels of sE-cad. In their study levels of sE-cad raised with age both in cancer patients and in healthy controls. From a revision of results of previous studies on the topic[40,41,43], they showed that these researches had been biased by not being matched for age patients and healthy controls[42].

Thus the cut off value was set on a lower value respect previous works: the optimal sensitivity (75.0%) and specificity (48.9%) were observed at a serum level of 4358.6 ng/ml[42]. The previous value of 10.000 ng/mL provided an higher specificity at the cost of a lower sensitivity.

Further studies could be of great value being the usual tumor marker, CEA, scarcely reliable as early predictor of recurrence.

CONCLUSIONS

Once excluded the specific issue related with familial GC where in the presence of suspected familial GC, after an anamnestic careful screening, CDH1 test may identify the subset of patients in whom an early prophylactic total gastrectomy could reverse a fatal destiny, E-cad show a particular clinical interest in three principal setting:

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