REVIEW

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Immunohistochemistry predictive markers for primary colorectal cancer tumors: where are we and where are we going?

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Abstract

The aim of our study is to highlight and organize the recently published immunohistochemistry (IHC) predictive biomarkers of primary colorectal cancers (CRCs) that could lead to practical implementation. We reviewed articles that examined CRC samples with significant statistic correlation between the IHC marker expression and disease progression over time, relationships with the available clinical features and those who detect the prognosis of drug effects. Our analysis showed that nine markers could correlate with medical treatment response of CRCs in different stages. When using better overall survival (OS) and better disease-free survival (DFS) as a grouping factor, there were 14 markers that could be used in assessing CRC prognosis. By using poor prognostic for the OS and the DFS as a grouping factor, we found 43 markers. Subgroup analysis was also performed based on the 32 markers recently confirmed to predict metastasis evolution or the recurrence risks. Venous invasion could be predictable for tumors, statistically significant metastasis susceptibility was observed for markers and also the capacity to evaluate recurrence. CRCs integrate a variety of localizations and there are proofs that distinguish the sites of tumors. The studies reporting data specifically for rectal cancer separating it from colon cancer contained seven IHC markers. In order to be able to implement a predictive biomarker in clinical practice, it must comply with certain criteria as clinical value and analytical proof. Unique biological signature of CRC can be distinguished by identifying biomarkers expression. Several markers have shown potential, but the majority still need to render clinical utility.

Keywords: immunohistochemistry, colorectal cancer, marker, prognostic, immunotherapy.

Introduction

The high incidence of colorectal cancer (CRC), one of the most common malignant tumors, ranks third among all cancer types [1]. The 5-year and 10-year survival rates for patients diagnosed with CRC are 65% and 58%, respectively. For the 39% of CRCs diagnosed at a localized stage, the 5-year relative survival rate is of 90% [2]. Clinical practice guidelines improved the effective results based on professional consensus. Obtaining a CRC tumor biopsy is nowadays a routine gesture and that transformed this type of malignancy into an ideal model for studying cancer's pathogenesis. In order to obtain an early diagnosis and to find particular features of tumors, the development of molecular medicine was stimulated and started to explore the biomarkers. A biomarker was defined as a characteristic that can be objectively measured and evaluated to assess a physiological as well as a pathological process or pharmacological response to a therapeutic intervention [3].

Handling tumor specific immune information has major effects on the therapeutically decision and patient's prognostic. An interruption in implementing the results into the clinical routine might be the absence of standardized procedures for most recent markers and the absence of a golden standard to evaluate the precision of results. Due to the impact on public health, although the progress is remarkable, immunotherapy associates with toxicity and their response rate urged to determine which patients would benefit the most from this kind of therapy. Nowadays, it relies on comprehending the molecular pathways in malignant cells, in order to obtain valuable predictive information. In the era of molecular biology, we can divide the markers in those which track disease progression over time and relationships with available clinical aspect and those which detect the effect of a drug [4]. The identification of immune biomarkers and their deciphering will fill in the knowledge gaps.

Consistent and reproducible results cannot be obtained by testing small number of biological samples, and the costs associated with the immunohistochemistry (IHC) studies can be prohibitive. In order to lower the costs, research groups fail into the trap of focusing on a restraint number of best hypotheses. This could lead to a fake track, which could end by missing the most accurate marker.

Our work systematically reviews the literature on the IHC biomarkers of primary CRCs that correlate with a predictive value concerning crucial particularities of this type of malignancy. The aim of our study is to highlight and organize the recent articles that could lead to practical implementation and targeted research.

Methodology

Published studies search and inclusion criteria

Articles were obtained by searching in the *PubMed* database issues from January 2012 up to June 30, 2017 with the following search syntax: "colorectal AND immunohistochemistry AND marker". The title and abstract of searched articles were assessed for a preliminary exclusion. We also checked the references of the retained articles in order to identify supplementary eligible status.

The results were reviewed according to the inclusion criteria consisting of (*i*) examination of CRC samples (including colon cancer or rectal cancer) with or without further supplementary analyses of lymph nodes or metastases; (*ii*) statistical evaluation of the relationship between IHC marker expression and prognosis, overall survival (OS), disease-free survival (DFS), response (RP) to chemoradiotherapy or recurrence (RC) or metastases tendency; (*iii*) the studies referenced in reviews which respected the same time period used in the search criteria.

The included texts were not limited to English language and among the eligible issues, there were also cohort studies and even randomized controlled trials. We excluded: (*i*) case reports; (*ii*) articles that lacked statistical significance in their observations ($p>0.05$). The inclusion process returned 65 articles.

Data extraction

The study data were extracted from each of the eligible articles: first author name, year of publication, number of cases analyzed, studied patients' particularities, the type of treatment, if the case and prognostic outcomes of interest (DFS, OS, RP, RC, and metastases tendency). For the categories where data were not reported in the study, the item was recorded as "0". Microsoft Excel 2010 (produced by Microsoft, USA) was used for data analyses.

Depending on prediction particularities, IHC markers were further classified into several categories: medical treatment response correlation, markers associated with lymphatic or distant metastasis and progression risks, localization related markers and relationship with patient OS and DFS.

Results from the reviewed studies

A total of 1893 studies were identified in the database search. Upon analysis of the titles and abstracts, 141 articles were reviewed. Of these, 76 studies did not involve statistic evaluation of prognostic related factors or data based on IHC technique results and were excluded. Sixty-five articles met all the inclusion criteria. The group contained 64 case control studies and one database review. Among the reviewed studies, there were seven articles containing results for rectal cancer, separately from all CRC tumors.

A total number of 81 different markers were found having significant statistical correlation with one or more of the classification categories used for data extraction. Most of them were related to OS and DFS and the number decreased for those correlated with treatment response or resistance.

Treatment response correlation

Our analysis showed that nine markers have the prognostic significance to correlate with medical treatment response for CRCs in different stages.

In order to evaluate the response to cetuximab treatment, Tural *et al.* [5] found phosphoinositide 3-kinase (PI3K) expression as a prognostic marker for resistance. In the same study of 41 *KRAS* wild-type metastatic (m) CRC patients who received second-line cetuximab- and irinotecan-based chemotherapy, it was found that the inactivation of phosphatase and tensin homolog (PTEN) predicted a bad response to cetuximab treatment [5]. An important article concerning several markers that could predict response to chemotherapy was published by Thomaidis *et al.*, in 2014. For the same tumor suppressor gene, PTEN, it was proven that high expression was a prognostic indicator for CRC patients undergoing irinotecan in addition with 5-fluorouracil/folinic acid (5-FU/FA) [6, 7]. Using tumors from 269 patients of the 5-FU/FA/irinotecan *versus* 5-FU/FA trial phase III of the *German Research Group Oncology of Gastrointestinal Tumors* (FOGT-4), for two of epidermal growth factor receptor (EGFR) ligands [8] negative expression, amphiregulin (AREG) and epiregulin (EREG), it was observed a longer OS and DFS from the addition of irinotecan in the adjuvant essential treatment [7]. For these EGFR-related markers, the best association for maximum survival under combined treatment was positive PTEN, negative AREG and negative EREG [8].

Using Mann–Whitney *U*-test, Ryan *et al.* showed that calnexin level was overexpressed in poor responders to neo-adjuvant radio-chemotherapy (RT-CT) (51 Gy and 5-FU) [9]. There were two articles that analyzed hypoxia-inducible factor 1-alpha (HIF-1α): one found that the over-expression was related with resistance to conventional chemotherapy [10, 11], and the other associated the negative expression with lower DFS for patients treated with irinotecan in addition to 5-FU/FA [6].

Wang *et al.* published a study concerning the heat shock protein 27 (HSP27) where shorter OS was found on a 62 patients subgroup with high expression that had received adjuvant chemotherapy with 5-FU [12]. For 84 advanced CRC tumors treated with oxaliplatin combined with fluoropyrimidines chemotherapy, with or without bevacizumab, better treatment response was found in patients with low expression of phosphorylated kinase domain receptor (pKDR) [13]. The important mechanism of hypoxic pathway seems to be involved in rectal cancer response to neo-adjuvant chemotherapy–radiotherapy and

high expression of glucose transporter 1 (GLUT1) predicts low rate of complete pathological response (ypCR) and poor treatment response [14]. All these markers are presented in Table 1 [6–16].

Table 1 – ***The distribution by name and year of the significant predictive markers correlated to medical treatment response in CRC using IHC technique***

Primary tumor marker	Description	No. of studies	No. of subjects	Year	References
PTEN	tumor suppressor gene acting as a downstream effector of EGFR	2	269 + 41	2014, 2014	[6, 7]
AREG	ligand of EGFR and activate the EGFR mediated intracellular cascade	1	269	2014	[7, 8]
Calnexin	ER stress protein	1	22	2016	[9]
EREG	ligand of EGFR and activate the EGFR mediated intracellular cascade	1	269	2014	[7, 8]
HIF-1*α*	a transcription factor that activates a large number of genes including VEGF	2	269	2014, 2016	[7, 10, 11]
HSP27	a molecular chaperone with anti-aggregation property involved in the proteasomal degradation of certain proteins under stress conditions	1	175	2012	[12, 15, 16]
PI3K	signaling pathway involved in cell proliferation, survival and motility invasion	1	42	2014	[5]
pKDR	the overexpression of the activated form of VEGFR-2	1	84	2016	[13]
GLUT1	involved in the hypoxic pathway	1	104	2013	[14]

AREG: Amphiregulin; CRC: Colorectal cancer; EGFR: Epidermal growth factor receptor; ER: Endoplasmic reticulum; EREG: Epiregulin; HIF-1*α*: Hypoxia-inducible factor alpha; GLUT1: Glucose transporter 1; HSP27: Heat shock protein 27; IHC: Immunohistochemistry; PI3K: Phosphoinositide 3-kinase; pKDR: Phosphorylated kinase domain receptor; PTEN: Phosphatase and tensin homolog; VEGF: Vascular endothelial growth factor; VEGFR-2: Vascular endothelial growth factor receptor-2.

Markers associated with metastasis and progression risk

Subgroup analysis was also performed based on the 32 markers recently confirmed to be associated with metastasis evolution or the recurrence risks. IHC analyze of tumor tissues found a majority that predict lymph node metastasis in CRC patients: positive expression of aldehyde dehydrogenase 1 (ALDH1) [17], absence of stromal CD276 (B7-H3) expression [18], overexpression of aquaporin 5 (AQP5) [19], presence of *BRAF* V600E mutation [20], cyclooxygenase-2 (COX-2) overexpression [21], high expression of doublecortin-like kinase 1 (DCLK1) [22], overexpression of chromosome segregation 1-like (CSE1L) protein [23], intense expression of *KRAS* [24], loss of Ku70 protein [25], loss of p53 tumor protein expression [25], positive expression of leptin [26], dehydrogenase/reductase 9 (DHRS9) low expression [27], high expression of fusin, also known as C-X-C chemokine receptor type 4 (CXCR4) [28] and S100 calcium-binding protein A4 (S100A4) [29], overexpression of phosphorylated mitogen-activated protein kinase kinase 5 (pMEK5) [30] and insulin-like growth factor-1 receptor (IGF-1R) [31], Notch homolog 1, translocation-associated protein (NOTCH1) receptor overexpression [32].

Venous invasion is predictable in tumors with nuclear presence of B7-H3 [33], S100A4 high expression [29], low expression of caudal-type homeobox transcription factor 2 (CDX2) [34] and E-cadherin [34]. The expression of CD8T [35] and CD45RO [35] are related to the absence of venous metastasis.

Distant metastasis susceptibility was observed when finding low expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [36], overexpression of mitogen/extracellular signal-regulated kinase kinase 5 (MEK5) [37], positive expression of T-box transcription factor 2 (TBX2) [38] and of leptin [26]. Hairy enhancer of split-1 (HES-1) expression was correlated with distant metastasis at diagnosis [39], and presence of tumor metastasis suppressor KAI1 predicted less distant metastasis for CRC patients [17]. A marker for reduced cellular proliferation and reduced invasion was BAG family molecular chaperone regulator 3 (BAG3), as low IHC expression [40].

A significant probability for developing liver metastases was detected for overexpression of cell division cycle 20 homolog (CDC20) [41], for a high expression for CXCR4 [28], positive expression of human tissue kallikrein-related peptidase 10 (KLK10) [42] and vascular endothelial growth factor receptor-3 (VEGFR-3) [43]. There was another study of VEGFR-3 where higher expression in the membrane and cytoplasmic expression was associated with lung metastasis, and lower expression was associated with liver metastasis [32].

Markers whose analysis could evaluate recurrence would play an important role for the follow-up of the patients. In this category are DHRS9 with a low expression [27], presence of TBX2 (p=0.025) [38] and high expression of GLUT1 [14], alpha-dystroglycan (*α*-DG) [44], and the mesenchymal–epithelial transition factor gene (*c-MET*) [45]. The aggressive phenotypes characterized by younger age were associated with COX-2 expression [21].

All these markers are presented in Table 2 [46–57].

Table 2 – ***The distribution by name and year of the significant predictive markers associated with metastasis and progression risk in CRC using IHC technique***

Primary tumor marker	Description	No. of studies	No. of subjects	Year	References
ALDH1	detoxify and metabolize various endogenous and exogenous aldehydes, as well as oxidize retinol to synthesize retinoic acid	1	204	2017	[17, 46]

Primary tumor marker	Description	No. of studies	No. of subjects	Year	References
AQP5	a family of small membrane transport proteins	1	40	2012	[19]
B7-H3 (CD276)	an immunoregulatory protein that belongs to the B7 family of T-cell co-regulatory molecules	2	277 + 389	2012, 2014	[18, 33, 47]
BAG3	interact with HSP70 to regulate physiology and pathophysiology	1	50	2016	[40]
BRAF V600E mutation	is a valine (V) to glutamic acid (E) switch at codon 600 caused by the c.1799T>A transversion	1	472	2017	[20, 48, 49]
CD45RO+ T-cells	immune cell markers	1	130	2014	[35]
CD8T	cytotoxic CD8T+ promote and stabilize the immunologic synapse between T cell and APC	1	130	2014	[35, 50]
CDC20	is an essential cofactor of the APC/C	1	244	2013	[41]
CDX2	homeobox gene	1	4020	2012	[34]
c-MET	the proto-oncogene which encodes the tyrosine kinase receptor for hepatocyte growth factor	1	135	2015	[45]
COX-2	catalyzes the synthesis of prostaglandins from arachidonic acid and its expression is enhanced by proinflammatory cytokines	2	213 + 770	2017, 2015	[21, 51–54]
CSE1L protein	or CAS, plays roles in apoptosis, cell survival, chromosome assembly	1	127	2013	[23, 55]
CXCR4	known as CD184, is an alpha-chemokine receptor specific for CXCL12	1	720	2014	[28]
DCLK1	is one kind of MAPs	1	101	2016	[22]
DHRS9	a member of the SDR family that converts retinol to retinal	1	163	2016	[27]
E-cadherin	the initial step in tumor invasion and metastasis is the break-up of adhesion junctions mediated by E-cadherin	1	4020	2012	[29]
GAPDH	essential regulator of glycolysis	1	62	2017	[36]
GLUT1	involved in the hypoxic pathway	1	104	2013	[14]
HES-1	plays an important role in maintaining neural stem cells and intestinal progenitor cells and regulating apoptosis	1	320	2015	[39]
IGF-1R	plays a critical role in development, proliferation, invasion and survival of cancer cells	1	213	2017	[31]
KAI1	a suppressor gene of tumor metastasis	1	204	2017	[17]
KLK10	kallikreins are a subgroup of serine proteases that co-localize to chromosomal region 19q13.4	1	62	2012	[42]
KRAS	gene with oncogenic potential	1	100	2013	[24]
Ku70	form the Ku heterodimer complex with Ku80	1	152	2015	[25]
Leptin	the obesity hormone	1	108	2012	[26]
MEK5	play an important role in cell proliferation and apoptosis	1	494	2016	[37]
NOTCH1 receptor	regulate cell proliferation, differentiation and apoptosis, as well as angiogenesis	1	105	2015	[32]
p53	tumor-suppressor protein	1	152	2015	[25]
pMEK5	play a critical role in regulating cell proliferation and apoptosis	1	350	2012	[30]
S100A4	a proinvasive gene expression	1	333	2013	[29]
TBX2	plays a critical role in embryonic development	1	119	2013	[38]
VEGFR-3	the main angiogenic protein known, increases vascular permeability and major inducer of lymphangiogenic signaling	2	672	2013	[32, 43, 56, 57]
α-DG	alpha (extracellular) unit of non-integrin adhesion molecule	1	137	2012	[44]

ALDH1: Aldehyde dehydrogenase 1; APC: Antigen-presenting cell; APC/C: Anaphase-promoting complex/Cyclosome; AQP5: Aquaporin 5; BAG3: Bcl2-associated athanogene 3; BRAF: B-type Raf kinase; CAS: Cellular apoptosis susceptibility protein; CD184: Cluster of differentiation 184; CDC20: Cell division cycle 20 homolog; CDX2: Caudal-type homeobox transcription factor 2; c-MET: Mesenchymal–epithelial transition factor gene; COX-2: Cyclooxygenase-2; CRC: Colorectal cancer; CSE1L: Chromosome segregation 1-like; CXCL12: C-X-C motif chemokine ligand 12; CXCR4: C-X-C chemokine receptor type 4; DCLK1: Doublecortin-like kinase 1; *α*-DG: Alpha-dystroglycan; DHRS9: Dehydrogenase/reductase 9; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GLUT1: Glucose transporter 1; HES-1: Hairy enhancer of split-1; HSP70: Heat shock protein 70; IGF-1R: Insulin-like growth factor-1 receptor; IHC: Immunohistochemistry; KLK10: Kallikrein-related peptidase 10; KRAS: Kirsten ras 2; MAPs: Microtuble-associated proteins; MEK5: Mitogen/extracellular signal-regulated kinase kinase 5; NOTCH1: Notch homolog 1, translocation-associated protein; pMEK5: Phosphorylated mitogen-activated protein kinase kinase 5; S100A4: S100 calcium-binding protein A4; SDR: Short-chain dehydrogenase/reductase; TBX2: T-box transcription factor 2; VEGFR-3: Vascular endothelial growth factor receptor-3.

Relationship between markers and OS and DFS

When using better OS and better DFS as a grouping factor, there were 14 markers that could be used for CRC prognostic (Table 3 [58–65]). Song *et al.* proved that patients with a strong expression of AT-rich interactive domain-containing protein 3A (ARID3A) had a better OS [58]. In a 2016 article, it was found that the overexpression

of CD15 antigen correlated with a better DFS [60]. In the same 300 patients group, CD3 antigen has been associated with better OS and DFS [60]. From the same molecular category, CD45RO antigen had a statistical relation with better OS [35, 59]. Koelzer *et al.* found that CD8T expression in 130 CRC tumors significantly related to a favorable OS [35]. Another immune cell marker over-expression, scurfin or forkhead box P3 (FOXP3), was associated with better OS and DFS [59].

In a study of 1800 patients, it was observed that those who had a strong expression of cell division cycle 7-related (Cdc7) had also a favorable OS [60]. For one of the epithelial growth factor receptors (EGFRs), the receptor tyrosine-protein kinase erbB-3 (HER3) was stated that an overexpression was observed in patients with better OS [61]. The negative expression of the transcription factor HIF-1α in 269 human CRC xenograft models was a better OS prognostic factor for DFS [10]. The presence of KAI1 protein in tumor tissue had a better OS prognosis [17]. Tural *et al.* found a better DFS for patients presenting PI3K expression [5].

Using Kaplan–Meier analysis (p=0.012) and the univariate analysis (p=0.018) for the patients with a low expression of protein-tyrosine phosphatase 1B (PTP1B), Chen *et al.* found that they had a better OS [62]. One of the members of RAS family, Ras-like nuclear protein (Ran) was found in a high expression in CRC patients with a better OS [64]. Vasohibin-1 overexpression, member of angiogenesis pathway, was correlated with better OS and DFS [65].

Table 3 – ***The distribution by name and year of the significant predictive markers for better OS and better DFS in CRC using IHC technique***

Primary tumor marker	Description	No. of studies	No. of subjects	Year	References
ARID3A	is a member of the ARID family of DNA-binding proteins	1	690	2014	[58]
CD15+	immune cell marker	1	300	2016	[59]
CD3+	immune cell marker	1	300	2016	[59]
CD45RO+	immune cell marker	1	300 + 130	2016, 2014	[35, 63]
CD8T	cytotoxic CD8T+ promote and stabilize the immunologic synapse between the T cell and the APC	1	130	2014	[35, 50]
Cdc7	a protein kinase implicated in cell division, cell cycle checkpoint mechanisms and cancer progression	1	1800	2015	[60]
FOXP3	immune cell markers	1	300	2016	[59]
HER3	a member of the human EGFR	1	365	2015	[61]
HIF-1α	a transcription factor that activates a large number of genes including VEGF *via* binding in its regulatory region	1	269	2014	[10, 11]
KAI1	a suppressor gene of tumor metastasis	1	204	2017	[17]
PI3K	signaling pathway involved in cell proliferation, survival and motility invasion	1	41	2014	[5]
PTP1B	an important regulator of signaling pathways involved in human diseases such as obesity, diabetes, and cancer	1	96	2014	[62]
Ran	a small G protein belonging to the Ras superfamily of small GTPases	1	287	2013	[63, 64]
Vasohibin-1	selectively induced by angiogenesis stimulators	1	132	2014	[65]

APC: Antigen-presenting cell; ARID3A: AT-rich interactive domain-containing protein 3A; Cdc7: Cell division cycle 7-related; CRC: Colorectal cancer; DFS: Disease-free survival; DNA: Deoxyribonucleic acid; EGFR: Epidermal growth factor receptor; FOXP3: Forkhead box P3; GTPases: Guanosine triphosphatases; HER3: Receptor tyrosine-protein kinase erbB-3; HIF-1α: Hypoxia-inducible factor alpha; IHC: Immunohistochemistry; OS: Overall survival; PI3K: Phosphoinositide 3-kinase; PTP1B: Protein-tyrosine phosphatase 1B; Ran: Ras-like nuclear protein; VEGF: Vascular endothelial growth factor.

When using poor prognostic for the OS and the DFS as a grouping factor we found 43 markers. The over-expression subgroup associated with poor OS contained the CD133 antigen [44, 66], CD44v6 [47, 67], cytoplasmic polyadenylation element-binding protein 4 (CPEB4) [68], cysteine-rich 61 (Cyr61) [69], focal adhesion kinase (FAK) [67], HK1 [70], HSP27 [12], immature colon carcinoma transcript-1 (ICT-1) [69], La-related protein 1 (LARP1) [71], leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) [72], MEK5 [37], pMEK5 [30], S100A4 [29], KH domain-containing, RNA-binding, signal transduction-associated protein 1 (KHDRBS1), also known as the Src-associated substrate in mitosis of 68 kDa (Sam68) [73], serine threonine tyrosine kinase 1 (STYK1) [74], proliferating cell nuclear antigen (PCNA) [71], phosphorylase kinase subunit beta (PHKβ) [75], tumor necrosis factor receptor-associated protein 1 (TRAP1) [76], tryptase [59], VEGFR-3 [32, 43], Nck-interacting kinase (TNIK) [77], transmembrane protease, serine 4 (TMPRSS4) [78], vasculogenic mimicry (VM) [17], wingless-type MMTV integration site family, member 7A (Wnt7α) [79], α-DG [44], β-catenin [29].

Also, a weak OS are expected when IHC-positive results are found for markers like ALDH1 (p<0.001) [17], *BRAF* V600E [49] and [20], HES-1 [39], KLK10 (for poor DFS, p=0.021) [42], Lgr5 [72], p42.3 [80], TBX2 [29].

There are markers with a low or a negative expression that correlate with a low OS like B7-H3 (CD276) [18], DHRS9 (for low DFS, p=0.003) [27], E-cadherin [29, 34], erythropoietin-producing hepatocyte (Eph) [81], histone H2B monoubiquitination (H2Bub1) [82], heterogeneous nuclear ribonucleoprotein K (hnRNP K) [12], karyopherin subunit alpha 2 (KPNA2) [83], Ku70 [25], O^6-methyl-guanine-DNA methyltransferase (MGMT) [84], p53 [25],

paired-like homeodomain transcription factor 1 (PITX1) [34].

The markers correlated with poor OS and DFS are presented in Table 4 [85–105].

Table 4 – ***The distribution by name and year of the significant predictive markers for poor OS and poor DFS in CRC using IHC technique***

Primary tumor marker	Description	No. of studies	No. of subjects	Year	References
ALDH1	detoxify and metabolize various endogenous and exogenous aldehydes	1	204	2017	[17, 46]
B7-H3 (CD276)	immunoregulatory protein in the B7 family	2	389 + 277	2014, 2012	[18, 33, 47]
BRAF V600E mutation	a serine/threonine kinase of the MAPK/ERK signaling pathway	1	188 + 472	2015, 2017	[20, 49]
CD133	a transmembrane glycoprotein related to cell–cell interaction and signal transduction, associated with cancer stem cells	2	123 + 137	2012, 2014	[45, 70, 84]
CD44v6	a polymorphic family of immunologically related cell–surface proteoglycans and glycoproteins	1	183	2013	[67]
CDC20	is an essential cofactor of the APC/C	1	244	2013	[41]
CPEB4	a zinc-finger-containing sequence-specific RNA-binding protein	1	393	2017	[68]
Cyr61	a member of the CCN proteins	1	251	2014	[69]
DHRS9	a member of the SDR family that converts retinol to retinal	1	163	2016	[27]
E-cadherin	the initial step in tumor invasion and metastasis is the break-up of adhesion junctions mediated by E-cadherin	2	333 + 4020	2013, 2012	[29, 34]
Eph	part of family of tyrosine kinase receptors in the human genome	1	124	2014	[81]
FAK	a transmembrane protein belonging to the family of non-RTKs, located at the focal adhesions	1	183	2013	[67]
HES-1	important role in maintaining neural stem cells and intestinal progenitor cells, balancing cell fate decision and regulating apoptosis	1	320	2015	[39]
H2Bub1	a potential tumor suppressor role	1	1800	2016	[82, 85–87]
HK1	is the first enzyme of glycolysis to be identified that couples cytosolic glycolysis to mitochondrial oxidative phosphorylation	1	622	2016	[70, 88]
hnRNP K	is an essential RNA- and DNA-binding protein that regulates diverse biological events	1	175	2012	[12, 89]
HSP27	a molecular chaperone with anti-aggregation property involved in the proteasomal degradation of certain proteins under stress conditions	1	175	2012	[12, 15, 16]
ICT-1	a component of mitochondrial ribosome (mitoribosome) and that it has PTH activity	1	861	2016	[69]
KLK10	kallikreins are a subgroup of serine proteases that co-localize to chromosomal region 19q13.4	1	62	2012	[42]
KPNA2	a member of the karyopherin alpha-family, also known as importin-α1	1	195	2015	[83]
Ku70	form the Ku heterodimer complex with Ku80	1	152	2015	[25]
LARP1	a RBP	1	40	2016	[71, 90]
Lgr5	a member of the GPCR family of proteins and is a target of Wnt signaling	1	192	2012	[72, 91–93]
MEK5	play an important role in cell proliferation and apoptosis	1	494	2016	[37]
MGMT	is a DNA repair protein that removes O^6-guanine adducts from DNA	1	123	2014	[84, 94]
p42.3	a direct target of miR-29a, which might act as a tumor suppressor in many kinds of tumors	1	212	2013	[80, 95]
p53	tumor-suppressor protein	1	152	2015	[25]
PCNA	the accessory protein of DNA polymerase δ plays an important role in cell proliferation, DNA replication and repair	1	40	2016	[71, 96]
PHKβ	is a serine/threonine protein kinase that phosphorylates and activates PYGL part of regulatory subunits	1	154	2017	[75, 97]
PITX1	homeobox gene	1	4020	2012	[34]
pMEK5	plays a critical role in regulating cell proliferation and apoptosis	1	350	2012	[30]
S100A4	a proinvasive gene expression	1	333	2013	[29]
Sam68	is originally identified as a substrate for Src kinase phosphorylation during mitosis	1	224	2013	[73]
STYK1	a new member of the RPTK-like protein family and found to be expressed in several normal human tissues	1	353	2015	[74, 98, 99]

Primary tumor marker	Description	No. of studies	No. of subjects	Year	References
TBX2	plays a critical role in embryonic development	1	119	2013	[38]
TMPRSS4	a newly discovered subfamily of serine proteases, emerging roles in carcinogenesis and progression	1	122	2013	[78]
TNIK	a member of the germinal center kinase family, essential for Wnt signaling and CRC proliferation and progression	1	220	2015	[77, 101, 102]
TRAP1	is a mitochondrial heat shock protein that has been related to drug resistance and protection from apoptosis in CRC	1	714	2017	[76]
Tryptase	immune cell markers	1	300	2016	[59]
VEGFR-3	the main angiogenic protein known, increases vascular permeability, a major inducer of lymphangiogenic signaling	2	105 + 672	2015, 2013	[43, 44, 56, 57]
VM	blood supply formation often seen in highly aggressive tumors	1	204	2017	[17]
Wnt7*α*	performs differential roles in tumor invasion and metastasis	1	212	2015	[79, 103–105]
α-DG	alpha (extracellular) unit of non-integrin adhesion molecule	1	127	2012	[44]
β-Catenin	nuclear translocation triggers an EMT	1	333	2013	[29]

ALDH1: Aldehyde dehydrogenase 1; APC/C: Anaphase-promoting complex/Cyclosome; BRAF: B-type Raf kinase; CCN proteins: ECM-associated proteins for intercellular signaling; CDC20: Cell division cycle 20 homolog; CPEB4: Cytoplasmic polyadenylation element-binding protein 4; CRC: Colorectal cancer; Cyr61: Cysteine-rich 61; DFS: Disease-free survival; *α*-DG: Alpha-dystroglycan; DHRS9: Dehydrogenase/reductase 9; DNA: Deoxyribonucleic acid; ECM: Extracellular matrix; EMT: Epithelial–mesenchymal transition; Eph: Erythropoietin-producing hepatocyte; FAK: Focal adhesion kinase; GPCR: G protein-coupled receptor; H2Bub1: Histone H2B monoubiquitination; HES-1: Hairy enhancer of split-1; hnRNP K: Heterogeneous nuclear ribonucleoprotein K; HSP27: Heat shock protein 27; ICT-1: Immature colon carcinoma transcript-1; IHC: Immunohistochemistry; KLK10: Kallikrein-related peptidase 10; KPNA2: Karyopherin subunit alpha 2; LARP1: La-related protein 1; Lgr5: Leucine-rich repeat-containing G protein-coupled receptor 5; MAPK/ERK: Mitogen-activated protein kinase/Extracellular signal-regulated kinase; MEK5: Mitogen/extracellular signal-regulated kinase kinase 5; MGMT: O^6-methylguanine-DNA methyltransferase; OS: Overall survival; PCNA: Proliferating cell nuclear antigen; PHK*β*: Phosphorylase kinase subunit beta; PITX1: Paired-like homeodomain transcription factor 1; pMEK5: Phosphorylated mitogen-activated protein kinase kinase 5; PTH: peptidyl-tRNA hydrolase; PYGL: Glycogen phosphorylase, liver form; RBP: RNA-binding protein; RNA: Ribonucleic acid; RPTK: Receptor protein tyrosine kinase; RTKs: Receptor tyrosine kinases; S100A4: S100 calcium-binding protein A4; Sam68: Src-associated substrate in mitosis of 68 kDa; SDR: Short-chain dehydrogenase/reductase; STYK1: Serine threonine tyrosine kinase 1; TBX2: T-box transcription factor 2; TMPRSS4: Transmembrane protease, serine 4; TNIK: Nck-interacting kinase; TRAP1: Tumor necrosis factor receptor-associated protein 1; VEGFR-3: Vascular endothelial growth factor receptor-3; VM: Vasculogenic mimicry; Wnt7*α*: Wingless-type MMTV integration site family, member 7A.

Localization related markers

CRCs integrate a variety of localizations and there are proofs that distinguish the sites of tumors. The studies reporting data specifically for rectal cancer or colon cancer contained seven IHC markers (Table 5).

The vasculogenic mimicry marker overexpression is frequent for colon tumors rather than rectal tumors and those patients have also a poor OS [17]. In an additional survival analysis performed by Song *et al.*, in the study of 388 patients with colon cancer and 302 patients with rectal cancer, the strong expression of ARID3A was significantly associated with a favorable outcome in patients with colon cancer, but not in patients with rectal cancer [58]. The absence of the angiogenic protein VEGF-C [56, 57] was frequent for tumors localized in colon and in a 672 patients study group it was observed that for stage III rectal cancer represented a poor OS prognostic [43]. Another marker associated with rectal cancer and large tumors size was hnRNP K studied on 175 primary CRCs and their corresponding normal mucosa [12]. In the same group, it was observed that positive expression of HSP27 was significantly related to colon cancer [12].

Concerning the laterality of tumor location, we found articles where it was proved that the presence of *BRAF* V600E mutation is specific to right sided tumors [20] and the c-MET proto-oncogene [45] is overexpressed in left colon and tumors larger than 5 cm [45]. Basic transcription factor 3 (BTF3), the RNA transcription factor, had a higher expression in distal cancers than in proximal cancers and his overexpression was significantly increased from in metastases [106].

Table 5 – ***The distribution by name and year of the significant predictive markers related to tumor localization in CRC using IHC technique***

Primary tumor marker	Description	No. of studies	No. of subjects	Year	References
ARID3A	a member of the ARID family of DNA-binding proteins	1	690	2014	[58]
BTF3	a general RNA polymerase II transcription factor and is also involved in apoptosis regulation	1	156	2013	[106]
BRAF V600E mutation	a valine (V) to glutamic acid (E) switch at codon 600 caused by the c.1799T>A transversion	1	472	2017	[20, 48, 49]
c-MET	the proto-oncogene which encodes the tyrosine kinase receptor for hepatocyte growth factor	1	135	2015	[45]
hnRNP K	is an essential RNA- and DNA-binding protein that regulates diverse biological events	1	175	2012	[12, 89]
HSP27	a molecular chaperone with anti-aggregation property involved in the proteasomal degradation of certain proteins under stress conditions	1	175	2012	[12, 15, 16]

Primary tumor marker	Description	No. of studies	No. of subjects	Year	References
VEGF-C	increases vascular permeability and is the main angiogenic protein known	1	672	2013	[43, 56, 57]
VM	blood supply formation often seen in highly aggressive tumors	1	204	2017	[17]

ARID3A: AT-rich interactive domain-containing protein 3A; BRAF: B-type Raf kinase; BTF3: Basic transcription factor 3; c-MET: Mesenchymal–epithelial transition factor gene; CRC: Colorectal cancer; DNA: Deoxyribonucleic acid; hnRNP K: Heterogeneous nuclear ribonucleoprotein K; HSP27: Heat shock protein 27; IHC: Immunohistochemistry; RNA: Ribonucleic acid; VEGF-C: Vascular endothelial growth factor-C; VM: Vasculogenic mimicry.

In order to understand oncogenesis and pathological mechanism, if we examine the markers by their position in tumors, there are interesting studies available (Table 6). The immunoregulatory protein B7-H3 was significantly associated with reduced recurrence-free survival in TNM stage I colorectal, but not with OS when located in nuclear position and predicted a vascular invasion [33]. Nuclear localization of Sam68 was associated with aggressive phenotypes and with poorer survival of CRC patients [73]. The nuclear localization of *β*-catenin was more specific for left-sided tumors compared to right-sided CRC [29].

Intra-tumoral endoglin (CD105) overexpression is associated with big tumor size and nodal invasion and the peri-tumoral pan-endothelial marker CD34 is statistically correlated with tumor size [107]. By studying 183 patients with CRC tumors, Garouniatis *et al.* found that CD44v6 can be used to predict the position of tumor for tumors bigger than 5 cm [67]. The high cytoplasmatic level of cellular apoptosis susceptibility protein, CSE1L, was associated with increased lymph node metastasis and increased disease recurrence [23].

Table 6 – ***The distribution by name and year of the significant predictive markers related to cellular localization in CRC using IHC technique***

Primary tumor marker	Description	No. of studies	No. of subjects	Year	References
B7-H3 (CD276)	an immunoregulatory protein that belongs to the B7 family of T-cell co-regulatory molecules	2	389 + 277	2014, 2012	[18, 33, 47]
CD105	or endoglin, has an expression only in the endothelial cells of the tumor blood vessel	1	31	2015	[107]
CD34	pan-endothelial marker	1	31	2015	[107]
CD44v6	a polymorphic family of immunologically-related cell–surface proteoglycans and glycoproteins	1	183	2013	[67]
Sam68	is originally identified as a substrate for Src kinase phosphorylation during mitosis	1	224	2013	[73]
β-Catenin	nuclear translocation triggers an EMT	1	333	2013	[29]

CD: Cluster of differentiation; CRC: Colorectal cancer; EMT: Epithelial–mesenchymal transition; IHC: Immunohistochemistry; Sam68: Src-associated substrate in mitosis of 68 kDa.

The genesis of the malignant process initiates after a series of limited genetic alterations in tumor suppressor genes and oncogenes. These facts started recently to be applied. Handling tumor specific immune information has major effects on the therapeutically decision and patient's prognostic. Obtaining a CRC tumor biopsy is nowadays a routine gesture and that transformed this type of malignancy into an ideal model for studying cancer's pathogenesis. When viable endoscopic material is provided, IHC technique manages to identify particular tumoral cell types for diagnosis purpose [100]. The surgical excision tissue of colorectal tumors provides a source of numerous samples. However, when only endoscopic or needle biopsies are usable, pathologists should optimize the use of specimens.

In colorectal malignancies, the connections of molecular changes produce biological interaction networks [108, 109]. Results from various banked specimens are heterogeneous and this is why important trials conserve only non-viable tumors or serum samples. Nowadays, biomarkers description arises after multiple assays of multiple tissues from multiple institutions using multiple industrial products. This multiple connection circuit influence the feasibility of rating systemic response. Biomarkers research procedures are complex and need first to identify the markers in a retrospective study, second to ascertain it in another confirmation set of patients and finally to validate it using randomized prospective studies. Only a couple of markers passed the validation procedures. Multifactorial analysis is acknowledged as a close up manner to assess the complex tumor molecular relationships aiming to detect key molecular pathways [109–111]. Reliable results and reproducible techniques will be obtained only after testing an important number of samples, but we should retain that immunoassays will associate with remarkable costs.

Generally, clinical practice guidelines improved the effective results based on professional consensus. An interruption in implementing the results into the clinical routine might be the absence of standardized procedures for most recent markers and the absence of a golden standard to evaluate the precision of the results. In order to be able to implement a predictive biomarker in the clinical practice, it must therefore be in accordance with certain criteria as clinical value and analytical proof. The need to obtain a suitable system led to elaboration of some guidelines for biomarker testing [112] with recommendations regarding sensitivity, specificity and reproducibility [113–115].

Advancing these ideas, research groups tried to determine the interplays between the peculiarity of local and circulating markers and tumor's immunology. The first study that confirmed the successful progress in tumor immunology and showed the potential of this kind of research proved that the design of tumor infiltrate has a

significant association with the outcome of CRC patients [116]. Strikingly, a report showed that the immunoscores obtained using central and peripheral tissue samples from CRC tumors had an improved prediction of survival than the well-known TNM staging [117, 118]. All this led to finding prognostic and predictive markers useful for diagnostic process, treatment choice, creating a research bridge between animal model studies and clinical expectation [119, 120]. Valuable biomarker research starts on a series of pathway approach that begins with the discovery phase and succeeds with clinical validation [113], if associated with biological processes and clinical endpoints [120, 121]. Markers evaluation concern on their capacity to estimate the outcome regardless of the treatment followed (prognostic value) and the capacity to assess the results and the side effects of a specific treatment (predictive). American *Food and Drug Administration* classified the biomarkers in three degrees: exploratory biomarkers, probable valid and known valid markers [113, 122]. Markers' validation method is different from pharmacokinetic confirmation and habitual laboratory validation. Using multiple proteins examination can improve the efficiency, but is limited due to the necessity to control evaluation conditions. Sensitivity will disappear reported to single assays and analyte's quality control will be different [123].

In tumor microenvironment, interactions similar to lymphocytic reactions occur [124, 125]. Various proofs revealed differences between rectal cancer, distal and proximal colon cancer [125–128] materialized in interference effects between tumor and microenvironment. Comparing the three entities, it was observed a higher incidence of microsatellite instability in proximal colon cancer [126]. The distal colon and the rectum are embryological derived from the hindgut while the proximal colon is derived from the midgut, both with different blood supply and their epithelial cells are exposed to different intestinal content [128]. Thus, biological markers correlation could differ by tumor location [109]. New conversance in CRC pathological evolution and treatment strategies can show up when unraveling correlations in biomarker networks [129, 130]. Some authors defined the hubs as highly connected molecular markers from cancer network that play key roles in oncogenesis [109, 131].

Overall outcomes for cancer treatment are significantly improved in targeted chemotherapy compared with empiric therapy. Nevertheless, there may exist tumors that are resistant to new generation chemotherapy from the beginning or may become after some cycles. Because tumors are usually progressing when drug resistance occurs, it would be indicated to evaluate the particularities of patients at treatment onset. The most part of markers are proteins used as substitute end-points for drug research, but in order to understand malignancy biology, diagnostic markers can be powerful. Several arguments require diagnostic tools to evaluate or to anticipate the response of each patient during therapy. Authors observed that low expression of tumor antigen correlates with a lack of immune response and changes in antigen presentation will provide a reduced cellular cytotoxicity action [132]. Sensitive and specific assessment should not be time dependent, expensive or exclusive, because the optimal moments that impose treatment changes can easily be missed. For example, imaging explorations are among the present options, especially the positron emission tomography scan, which cannot be ordinarily used to monitor the therapy efficiency because of costs, radiation and toxicity.

Oncological teams analyzed the intensification of adjuvant treatment for colorectal patients. They reported that neoadjuvant therapy for rectal cancer correlate with OS with a complete pathological response that varies between 10% and 49% [118, 132, 133] and the use of complementary oxaliplatin with 5-FU has a pathological response rate similar to those who benefited of additional cycles of neoadjuvant therapy before the surgery [134–136]. Other studies investigated the differences between the alternatives concerning radiotherapy administration and found thaat the long course procedure associated with improved pathological down staging rates and more frequent complete pathological response rates [137, 138]. In stage III colon cancer, adjuvant chemotherapy is a standard procedure. Variability in drug response and side effects are hidden features of treatment and maintain the debate if whether post-surgical drugs should be routinely administered in stage II patients. Important speculation, as EGFR overexpression analyzed by IHC could forecast the response to anti-EGFR treatment, were initiated without being strengthened by studies [139, 140]. On the other way, the several published paper related to tumoral markers, only *KRAS* mutation and *UGT1A1* 28 polymorphism have integrated into the therapeutically decision part [139].

The heterogeneity of rectal tumors can also be observed in the variable rates of complete therapeutically response pronounced in different studies in spite of analogous neo-adjuvant treatment procedures [118]. A noteworthy aspect for assessing the prediction of therapy response is the time interval between neoadjuvant treatment and surgical intervention. There is a chronological correspondence between the rate of complete therapeutically response and radiation induced apoptosis. Authors published higher rates of complete response after a longer time gap between the end of chemo-radiotherapy and surgical operation [118, 140–142], but recent studies show that the delay should not be longer than seven weeks [129]. Biomarkers capacity to describe the response to radiotherapy can create ways for selecting the patients whom would benefit from exclusive or neoadjuvant intensive radiation cycles. Drugs designed to block the markers involved in the molecular pathways of malignant cells can be included into the treatment bases. Personalizing the treatment according to the probability of tumor response will balance the potential risks and will improve the outcome of patients diagnosed with rectal cancer [109]. Since targeted therapy is used, metastatic CRC patients have a prolonged OS [143].

Nowadays, the most important prognostic factor is the pathological stage. Due to conflicting evidence and lack of multivariate analyses, oncological societies do not use many markers for prediction or prognosis. Available are the carcinoembryonic antigen recommended quarterly for at least three years for stage II and III CRC [144, 145] and *KRAS* mutation testing for validation of EGFR-based

treatment [146, 147], but the last one is not recommended in surveillance or monitoring the treatment response [147]. For patient with primary carcinoma of the colon and the rectum, there are reported guidelines that suggest to include IHC protein expression from DNA mismatch repair genes *MLH1*, *MSH2*, *MSH6* and *PMS2* with molecular markers of *MSI*, *KRAS* and *BRAF* gene mutations [112]. The majority of markers are studied in the signaling pathways EGFR/Ras/Mek/Erk and PTEN/ PI3K/AKT [139].

The results of the study might have been influenced by certain bias. Even if we analyzed large-scale recognized IHC markers of CRC pathogenesis, we could not include a majority of known markers. This is an inevitable restriction in this kind of study as it was limited by selection criteria.

Despite these limitations, our work has several strengths that distinguish it from previous reviews. It focuses on valuable recent progress concerning prognostic biomarkers based on different aspects as survival, medical treatment response and aggressiveness of CRC tumors. Various features of colorectal malignant tumors are now cross-examined for markers' application and therapeutically implementation. The originality of this work is represented mainly by the principal inclusion criteria for the articles in the reviewed group, namely the statistical confirmation of markers' evaluation capacity. Furthermore, the results materialized in an updated register of noticeable CRC markers detectable using the IHC technique. The quest for prognostic and predictive biomarkers has allowed advancing out those who could be used for further studies from now on.

Conclusions

The last half decade of CRC research has produced an important amount of results. In order to personalize the treatment for CRC patients it is necessary to understand its natural history and malignant genesis mechanisms that help the disease progress. Unique biological signature of CRC can be distinguished by identifying biomarkers expression. Several markers have shown potential, but the majority has not yet rendered clinical utility. Further large prospective studies and individualized approach studies based on particular disease characteristics will pave the way for personalized medicine. For each disease stage apart, therapeutic management based on biomarkers testing results will allow better use of health care resources and may relieve the patient of unworthy procedures.

Conflict of interests

The authors declare that they have no conflict of interests.

Author contribution

Alexandru Bărbălan and Mircea-Sebastian Şerbănescu had equal contributions.

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