[Frontiers in Bioscience 19, 312-338, January 1, 2014]

Molecular diagnostics in gastric cancer

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TABLE OF CONTENTS

1. Abstract
2. Introduction and epidemiological background
3. Developments in endoscopy
4. Histological assessment
    4.1. Histopathology of gastritis and attributable risk
    4.2. SPEM - spasmolytic polypeptide expressing metaplasia
    4.3. Subtyping of mucins
    4.4. Expression of Her2
5. Serological biomarkers
    5.1. Pepsinogens and the "serological biopsy"
    5.2. Cancer autoantibodies
    5.3. Circulating cancer cells
6. Volatile organic components in the breath
7. Molecular diagnostics
    7.1. MicroRNA
    7.2. Epigenetic changes
    7.3. Genetic alterations
8. Biostatistical assessment
9. View to the future and conclusion
10. Acknowledgements
11. References

1. ABSTRACT

Despite recent advances in individualised targeted therapy, gastric cancer remains one of the most challenging diseases in gastrointestinal oncology. Modern imaging techniques using endoscopic filter devices and *in vivo* molecular imaging are designed to enable early detection of the cancer and surveillance of patients at risk. Molecular characterisation of the tumour itself as well as of the surrounding inflammatory environment is more sophisticated in the view of tailored therapies and individual prognostic assessment. The broad application of high throughput techniques for the description of genome wide patterns of structural (copy number aberrations, single nucleotide polymorphisms, methylation pattern) and functional (gene expression profiling, proteomics, miRNA) alterations in the cancer tissue lead not only to a better understanding of the tumour biology but also to a description of gastric cancer subtypes independent from classical stratification systems. Biostatistical means are required for the interpretation of the massive amount of data generated by these approaches. In this review we give an overview on the current knowledge of diagnostic methods for detection, description and understanding of gastric cancer disease.

2. INTRODUCTION AND EPIDEMIOLOGICAL BACKGROUND

Taken into consideration the predicted growth of the world population and the increase of average life expectancies in many countries, the absolute number of gastric cancer cases is likely to be stable or will even increase in the future despite a declining incidence (1). Almost one million cases are newly diagnosed each year, and about 740,000 deaths are caused by this disease resulting in 8% of all cancer cases and 10% of all cancer-related deaths annually (2). There is a regional variation of the incidence rates by the factor 10, and more than 70% of gastric cancers occur in developing countries due to higher *H. pylori* prevalence rates (2).

In contrast to a decline in the incidence of distal gastric cancer there has been an increase of adenocarcinomas at the oesophagogastric junction including gastric cardia cancer, mainly in North America and Europe(3-6). In Asia, distal gastric cancer still remains the main entity.

At the time of diagnosis of gastric cancer, there is usually only a short period of symptoms such as

unintentional weight loss, anaemia, epigastric pain, nausea and vomiting, or dyspeptic symptoms. About 40% patients don't complain about any dyspeptic symptoms at any time (7). Therefore, diagnosis for the majority of patients is made at an advanced stage when only limited treatment options can be offered. Even though there has been some improvement in the clinical management of gastric cancer, the 5 year survival rate is <30% in most countries and the reported mortality rates mirror the incidence of the disease. Therefore, tools to enable an early diagnose are of critical importance.

3. DEVELOPMENTS IN ENDOSCOPY

In the absence of appropriate blood-based diagnostic tests for gastric cancer, upper gastrointestinal endoscopy with biopsy sampling for histopathological evaluation remains the gold-standard for determination of a definite diagnosis. The diagnostic yield of traditional white light endoscopy (WLE) can by enhanced by chromoendoscopic techniques (e.g. staining with methylene blue or indigo carmine) or application of acidic dye (8, 9).

Technical advances that allow virtual chromoendoscopy by the use of optical filter systems in combination with optical magnification of the mucosal surface pattern have nearly replaced the classical application of intraluminal dye (10).

Narrow band imaging (NBI) uses special wavelength filters for red coloured structures (e.g. blood vessels or inflamed areas) by which the detection of even early neoplastic lesions in the stomach can be dramatically increased compared to traditional WLE resulting in sensitivity and specificity above 90% (11-14).

The evaluation of the microvascular pattern as well as of the superficial structures allows the differentiation of small elevated lesions between adenomatous and cancerous tissue formations with higher accuracy, sensitivity and specificity for NBI compared to WLE(14-16). This approach can also be used for surveillance after endoscopic resection to detect residual or recurrent disease(17).

The mucosal pattern in magnifying NBI-enhanced endoscopy can give some indication for the depth of invasion in case of a malignant lesion (18). By evaluation of surface and microvessel structure it has even been possible to distinguish between Sm1 (cancers with intramucosal and minute submucosal invasion <500 µm in depth) and Sm2 (deeper submucosal invasion ≥500 µm in depth) early gastric cancer, (19). Similar criteria have been applied as surrogate parameters for the degree of differentiation of an early gastric cancer (20).

During the past years there has been strong effort to develop a general classification system for NBI-documented characteristics mainly focusing on the surface appearance including the gastric pit pattern, the microvascular structure as well as colour and shape of the respective lesion (21). The diagnostic quality of these patterns were satisfying although interobserver variation was still high (13, 22). Compared with adenomas, carcinomas present at bigger size, depressed morphology, red colour and positive findings (irregularities) in surface and vessel structure (23-25).

One approach to improve the diagnostic quality of NBI-based endoscopic imaging is the combination with other filter systems. Autofluorescence imaging (AFI) has been introduced more than a decade ago. Despite a generally lower resolution compared to WLE, the different autofluorescent pattern in case of metaplastic or dysplastic lesions increased the diagnostic yield of targeted biopsies for dysplastic or neoplastic changes (26-28). However, it has been stated early, that AFI is of limited quality if used as single technique (29). Thus, the combination of a high resolution magnifying endoscope with AFI and NBI filter systems was introduced as so-called Trimodal Imaging Endoscopy (TME) for a direct assessment of AFI-positive lesions NBI imaging including a magnifying zoom technique to increase the sensitivity, specificity and accuracy of endoscopic diagnostic procedures (30). However, the final diagnostic value of the increased rate of targeted biopsies still needs to be evaluated (31, 32).

A similar approach to NBI represents the flexible spectral imaging colour enhancement endoscopy (FICE), which offers more than one optical filter in different digital channels that can be easily switched during the investigation. Each mucosal structure can be assessed best by certain channels resulting in high interobserver agreement (33). Furthermore, the high contrast enhancement allows a better definition of the lateral demarcation line of gastric lesions compared with other approaches, especially for depressed lesions (34, 35)(Figure 1).

The most recent technique is confocal laser endoscopy (CLE) that enables an *in vivo* real-time assessment of the histopathological alterations present in the gastric mucosa. First classifications of the related pit patterns have been developed on surgical specimens from gastric cancer patients and then validated in healthy volunteers (36). *In vivo* differentiation between physiological tissue from different regions of the stomach was possible as well as to distinguish between IM, non-metaplastic glandular atrophy and neoplastic lesions (36). This high-end magnification technique allows the detection of goblet cells and of a respective brush border in an absorptive intestinalised epithelium in case of intestinal metaplasia (IM) with a sensitivity about 90% and a specificity even higher (37, 38). Even the assessment of epithelial barrier function in response to *H. pylori* eradication can be assessed (39). However, the diagnostic accuracy and gastric cancer detection rate for CLE is significantly higher for experienced compared to inexperienced examiners since the investigation is time-consuming and demands high concentration and patience (40). In experienced hands – or better with experienced eyes - the diagnostic accuracy for the discrimination of cancerous lesions or even high grade intraepithelial neoplasia can be as high as 98.8% and by this comparable

to the classical histopathological evaluation (41, 42). This

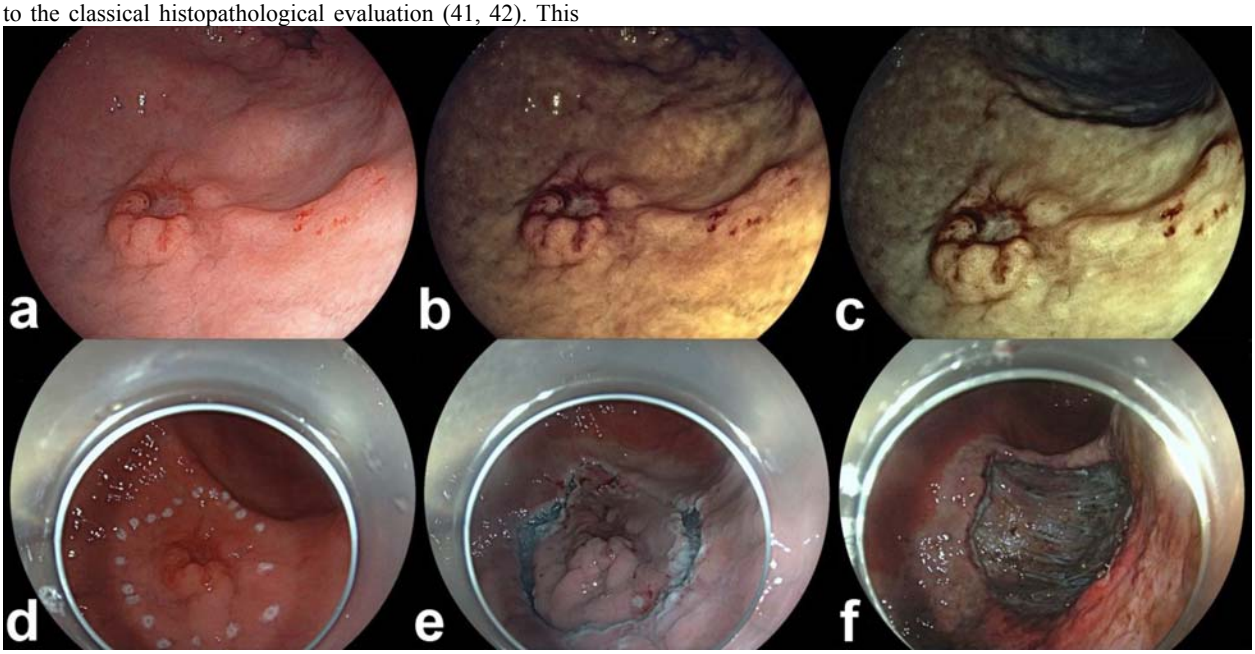

Figure 1. FICE imaging of an early gastric cancer before and after endoscopic resection. a-c) The images show and early gastric cancer of the intestinal type with central ulceration. The different FICE filter channels are used for better demarcation of the lateral tumour margins. d) Demarcation of the planned resection zone with an argon plasma beamer. e) Circumferential incision of the dissection zone by a hook-knife down to the level of the *submucosa*. f) Complete histopathological confirmed R0 resection of the intramucosal cancer. In view are the muscle layers of the *muscularis propria*.

approach has further value in the assessment of resection margins after endoscopic treatment of early gastric cancer, there being even superior to biopsy sampling and histopathological assessment (43, 44). Another approach for the future could be the *in vivo* microscopy after labelling of the gastric mucosa with cancer specific fluorescent agents (45).

These technical advances can also be applied for diagnosis and risk stratification of premalignant conditions of the gastric mucosa like intestinal metaplasia or atrophic gastritis. For NBI, the appearance of a light blue crest sign is a valuable surrogate for the presence of IM (46, 47). The combination of an optical filter system with CLE can even enable "real time histology" that could reduce the number of biopsies that are necessary for the diagnosis of IM or atrophic changes (48).

However, despite the exciting development in the field of diagnostic gastrointestinal endoscopy, this does not allow so far to replace routine standard biopsy work-up and thorough inspection by standard white light endoscopy (49, 50). So far, new optical systems are only of practical value in specialised high volume centres in Asia, i.e. in regions with high incidence of gastric cancer.

4. HISTOLOGICAL ASSESSMENT

4.1. Histopathology of gastritis and attributable risk

The up-dated Sydney classification of gastritis with its updates is still the most widely used system for characterizing the status of the gastric mucosa either for research purposes or clinical practice. It combines topographic, morphological, and etiological information (51). The analysis of five biopsies – two from the antral part, one from the angulus, and two from the gastric body - is required to characterise gastritis in the absence of any visually detectable lesions. In addition to glandular atrophy and IM, each graded according to a visual analogue scale (0: no changes, 1: minor changes, 2: moderate changes, 3: severe changes), the classification requires also the reporting on the presence of *H. pylori*, active and chronic inflammation, the latter by the degree of mucosal infiltration by neutrophile granulocytes and lymphocytes, respectively.

In the past decade new classification systems have been developed to enable more consistent stratification of the individual gastric cancer risk attributable to the present preneoplastic changes of the gastric mucosa. OLGA and OLGIM staging systems for gastric premalignant lesions aim to simplify the clinical approach while using the same biopsy work-up as for the Sydney system. The abbreviation OLGA stands for "Operative Link on Gastritis Assessment" and is based on the assessment of glandular atrophy, while OLGIM emphasizes the importance of Intestinal Metaplasia.

The initially proposed OLGA system is based on pooling the atrophy stages in each part of the stomach into a simple scoring system ranging from "0" to "IV" (52). The stage by itself does not allow to judge the topography of the lesion revealed (in particular for the lower stages), but it indicates the individual likelihood to develop malignant

neoplasia since most of the cancer cases are expected to develop in patients who present with stages III and IV (53). In addition, the stage distribution is convenient also for research purposes, like the correlation of the histopathological OLGA score with serological biomarkers (54).

Considering that the interobserver agreement is better for assessment of IM than of non-metaplastic glandular atrophy, the OLGIM system is using the same approach, but classifying the degree of IM instead of assessing atrophic changes with or without metaplastic transformation of the gastric mucosa (55). It is still under debate if one of these approaches is superior to the other, resulting in a higher sensitivity for gastric cancer risk assessment (55-57). OLGA based stratification of premalignant lesions in the stomach can also be associated with non-atrophic mucosal alterations and certain inflammatory conditions (58, 59).

4.2. SPEM – spasmolytic polypeptide-expressing metaplasia

Spasmolytic polypeptide-expressing metaplasia (SPEM) is markedly characterised by an induction of the gene expression of the spasmolytic polypeptide which has been identified as the trefoil factor 2 (TFF2) (60). SPEM is mainly associated with corpus predominant gastritis and has been shown to be associated with gastric cancer development (61, 62). In surgical specimens from patients with early gastric cancer there was positive evidence of SPEM in the tumour surrounding mucosa if the tumour was located in the gastric body or at the body-antrum junction (63). In three quarters of the cases there was also SPEM in the tumour-distant body mucosa. TFF2 could be detected in 76% of dysplastic cells. In samples from a control cohort with gastritis and without neoplastic lesions, 82% of patients who developed gastric cancer during follow-up were positive for SPEM compared to 37% in cases without malignant transformation (63). Similar results are reported for the mucosa of patients with remnant adenocarcinoma after limited resection of gastric cancer (64).

Data from animal experiments suggested that the metaplastic cells derive from gastric Chief cells or alternatively develop by activation of basal crypt progenitor cells (65). However, recent data has shown, that these leucine-rich repeat containing G-protein-coupled receptor 5 (Lgr5) positive gastric stem cells are not the origin of SPEM (66, 67). Signalling cascades leading to the induction of these metaplastic changes seem to involve PGE2-related pathways and even Wnt-dependent signals (68).

4.3. Subtyping of mucins

The differential expression of mucin (MUC) subtypes marks the phenotype of gastrointestinal epithelium and allows the differentiation of physiological or metaplastic mucosa. There is physiological expression of MUC5AC in the superficial epithelium and the upper part of the gastric pits, as well as MUC6 in the lower part of the gastric glands (69). Further gastric-type mucins are MUC1 and the human gastric mucin (HGM). MUC2 is mainly expressed by goblet cells and characterises intestinal epithelium (69). Induction of the intestinal transcription factor CDX2 in the gastric mucosa leads to an upregulation of MUC2 gene expression and is mostly accompanied by a downregulation of the gastric transcription factor SOX2 mirrored by decreased MUC5AC secretion (70, 71).

These processes can be a response to *H. pylori* induced chronic inflammation since the degree of the lymphocellular infiltration is concordant with the intramucosal level of MUC2 expression and the pro-inflammatory cytokines *TNFα* and *IL1β*are capable of MUC2 gene induction (72). In contrast, gastric type mucins are significantly lower expressed in the gastric body of *H. pylori* positive patients, but show increasing levels after eradication therapy (73, 74). Lower expression is maintained in case of present mucosal transformation towards atrophic changes, dysplasia and gastric cancer.

According to the mucin expression profile, adenocarcinomas in the stomach can be classified into a gastric, an intestinal, and a gastrointestinal (mixed) phenotype (75, 76). The related mucin phenotype can alter in relation to the status of *H. pylori* infection. After eradication of *H. pylori* the mucin expression profile in the tumour tissue shows more often the gastric predominant type, whereas in non-eradicated patients the intestinal type is more present being associated with a less favourable prognosis (76-78).

Atypical mucins like MUC13 can be detected in 90% of IM, and MUC13 also upregulated in gastric cancer, mainly in intestinal type adenocarcinomas (79, 80).

Interestingly, the phenotype of present IM has not to be related to the histological type of the cancer, and intestinal type gastric cancer shows even often a gastric mucin profile (81, 82). There are still conflicting results concerning the correlation of mucin expression with clinicopathological parameters (83-87).MUC1 expression in the tumour centre correlates with advanced TNM-stage and lymph node involvement, and expression at the invasion front of the cancer represents an independent predictor for worse prognosis in multivariate analysis (88). MUC1 expression is lost during dedifferentiation of gastric tumours and most studies confirm a positive association to distant metastases, accompanied in most cases also by a decrease of MUC5AC gene expression (82, 89).

A recent genome-wide association study revealed single nucleotide polymorphisms (SNPs) of the MUC1 promotor to be involved in differential regulation of this mucin and therefore to be related to gastric carcinogenesis, mainly of the diffuse type (90). SNPs in the MUC1 gene have already been reported to be related to gastric cancer, whereas SNPs in the MUC5AC gene have only a minor impact (91). In contrast, polymorphisms of the MUC2 gene are associated with a decreased risk of progression of premalignant changes in the gastric mucosa, also interfering with the probability of regression after *H. pylori* eradication therapy (92). MUC2 gene expression can

also be epigenetically regulated by hypermethylation of its promotor region (93).

In a minor proportion of gastric cancer patients mucins can be detected in the peripheral blood as indicator for an induced expression. Surrogates like anti-MUC1 IgG in the peripheral blood are not constantly present and have therefore no value for clinical practice (94-96).

4.4. Expression of HER2

In the era of individualised targeted therapy of malignant diseases there has been a recent breakthrough for the treatment of gastric cancer when the results of the ToGA trial have been published, a prospective, randomized, placebo-controlled phase III trial on almost 600 patients with cancer of the stomach or at the oesophagogastric junction (97). In these patients with positive expression of the HER2 molecule, trastuzumab, a monoclonal antibody against HER2, has been administered in combination with standard cisplatin/5-fluorouracil based systemic chemotherapy. Mean overall survival of patients receiving the trastuzumab combination has been 13.8 months compared to 11.1 months in the placebo group (cisplatin/5-FU alone). Since this difference was not statistically significant, the major impact of the study was given, when a subgroup analysis revealed that in patients with strong expression of HER2 (IHC 3+ or IHC2+/FISH+) survival was as high as 17.9 months being highly significant compared to the placebo group (97).

HER2 can be detected in 10.1% to 20.6% of patients with gastric adenocarcinoma with higher positivity in intestinal type tumours and in patients with distant metastases (97-102). Results concerning HER2 expression as prognostic indicator are still conflicting with studies showing partly poorer and partly improved survival for HER2 positive patients (99, 100, 103-105). A systematic review on 42 publications including 12,749 patients reported in 71% of the trials an association of positive HER2 status with poor survival, serosal invasion, positive lymph node status, distant metastases, and more advanced stage of the disease (106). In another comprehensive review on 35 studies assessing survival, 20 could not demonstrate a survival benefit, two reported longer and 13 even shorter survival in HER2 positive patients. Overall five-year survival was 42% *vs.* 52% in favour of HER2 negative patients. However, the introduction of trastuzumab-based treatment regimens results in a clear survival advantage for HER2 positive patients (103).

HER2 expression is increasing with severity of mucosal alterations from lowest in low grade IEN and highest in adenocarcinoma of the stomach, even showing further increase with dedifferentiation of neoplastic lesions (107). Since there is high intratumour heterogeneity (Figure 2), there is an on-going debate whether biopsy sampling during endoscopy is adequate for assessment of HER2 status, and if tissue from metastatic sites (e.g. liver) can be used for estimation of the primary. As of today, it can be stated that there is high concordance between HER2 expression in the primary tumour and distant metastases as well as affected lymph nodes (101, 108-110). There is also no significant difference in the diagnostic quality if tissue from surgical resection specimens or endoscopic biopsies were used (101, 110, 111). However, these data need still further validation in clinical practice.

Since molecular testing and quantification of a positive test result has a significant value for the treatment decision there is still debate which test method presents the best diagnostic yield. Fluorescence *in situ* hybridisation (FISH) is superior to classical immune-histochemical staining (IHC) although it is more complicated and more expensive (112). Another option is dual-colour silver chromogenic ISH (CISH/SISH) that reveals even more precise test results and a higher detection rate than FISH (98, 99, 112). Tissue microarrays have been discussed to further enhance the diagnostic yield (100, 104, 113, 114).

HER2 expression in gastric cancer might even correlate with HER2 detection levels of the serum of the patients, but it is doubtful if these marginal changes render a benefit as a clinical biomarker for gastric cancer screening (115).

5. SEROLOGICAL BIOMARKERS

5.1. Pepsinogens and the „serological biopsy“

Pepsinogens are pro-enzymes of pepsin. Pepsinogen I (PgI) is exclusively produced by the chief and mucous neck cells of the corpus, while pepsinogen II (PgII) is also secreted by cardiac, pyloric and Brunner gland cells (116). Pepsinogen levels are decreased if atrophy occurs in the gastric body, while an increase is observed during inflammation. To reduce the possibility of false normal results at the occasion when both atrophy and *H. pylori* caused inflammation co-exist, the ratio between PgI and PgII (PgI/II) is considered a more reliable marker than PgI alone (117-119).

A comprehensive meta-analysis published in 2006on more than 40 studies including about 300,000 individuals suggested the rationalle of using pepsinogen testing to identify individuals at high risk to develop gastric cancer who would need further diagnostic work-up (118). Decreased pepsinogen levels yield a sensitivity of 66.7-84.6% and a specificity of 73.5-87.1% for the detection of atrophic gastritis (120-123). However, lower sensitivity (36.8%-62.3%) has been reported for direct gastric cancer screening even with similar cut-off values (124-126).

Considering confounding factors in gastric cancer patients, that could be included in a further stratified analysis,would be a valuable approach to modulate outcome of the pepsinogen test (127). However, neither Laurén type, nor tumor localisation or tumor stage have an influence on the serum values for PgI, PgII and PgI/II (127, 128). Only for the PgI/II a significant difference between intestinal and diffuse type carcinomas is documented in some studies which is related to the higher incidence of intestinal metaplasia and glandular atrophy in case of intestinal type cancers (129, 130).

Amidated gastrin-17 (G-17) has been suggested as additional marker to characterise atrophy in the antral

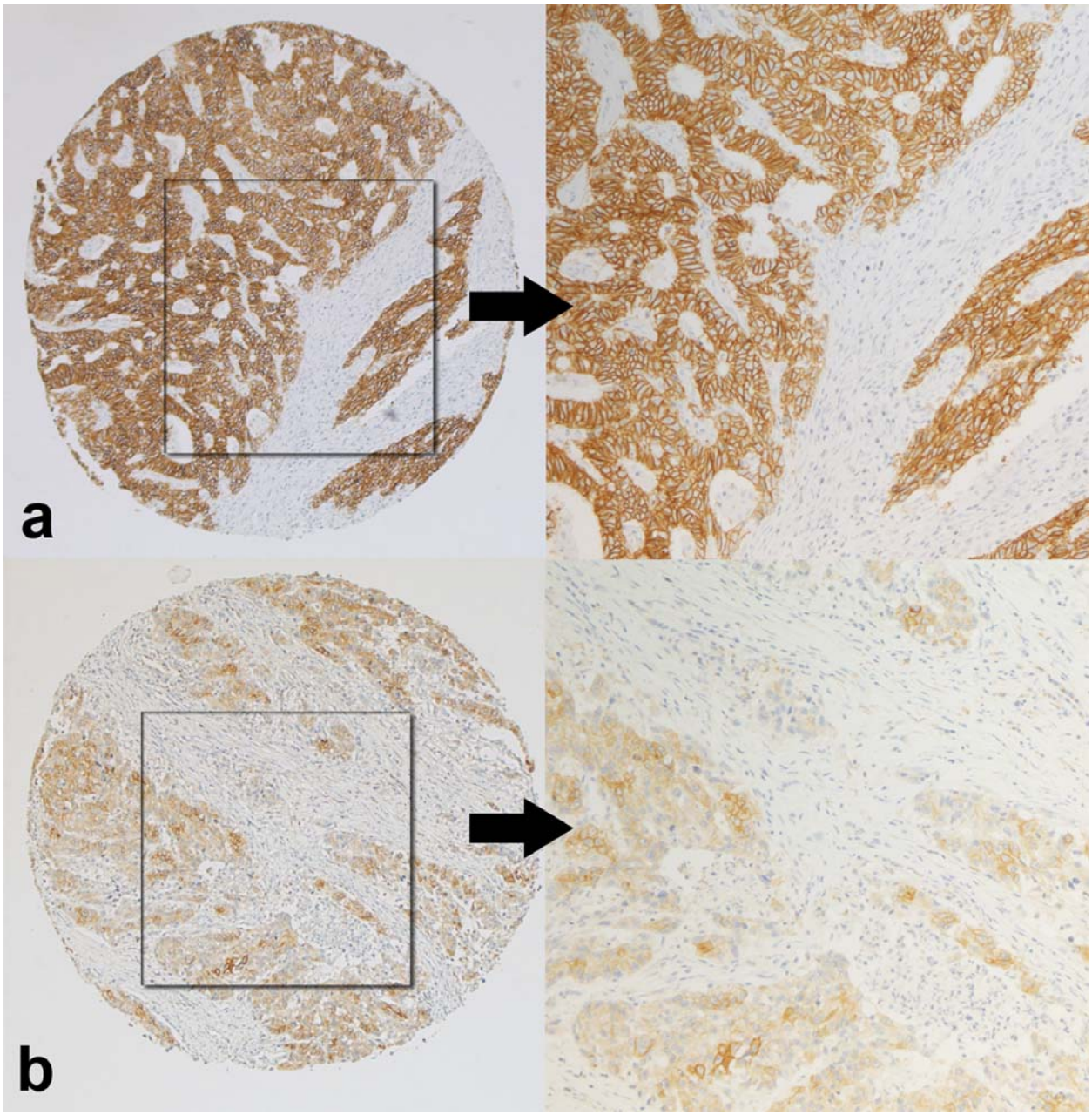

Figure 2. Representative examples of Her2 immunohistochemical staining. a) Strong and complete membranous Her2 staining with homogenous distribution throughout the sample (IHC-score 3+). b) Heterogenous distribution of Her2 staining, IHC-score 2+; FISH analysis is recommended. Original magnifications 100x, 200x.

part of the stomach. G-17 is a sub-fraction of total gastrin consisting of 17 amino-acids that is secreted exclusively by the G-cells in the gastric antrum (131-133).G-17 levels in the serum can indicate two conditions: decreased levels could be indicative for atrophy in the antral part of the stomach, but increased are characteristic for corpus atrophy (further characterised by low pepsinogen levels) in the absence of atrophy in the antral part. G-17 levels in the circulation are increased after food intake; therefore the measurements of G-17 following a provocation with a protein-rich meal are considered the best indicator of the function of antral G-cells (132, 134). However, due to the limitations of this provocation test (e.g. repeated blood sampling since other markers are usually analyzed at a fasting state, intake of a test-meal, duration of the test), in many studies only single measurement at fasting state is performed (116, 135).

A false positive increase of G-17 could be feedback to drug-induced hypochlorhydria in the stomach or inflammation, and a decrease could be related to acid peptic disease (133, 136-138). Therefore, despite a good

specificity of decreased G-17 for diagnosing antral atrophy in Caucasians (91.5% at a fasting state and 92.6% after stimulation), the sensitivity is unsatisfactory (15.4% at a fasting state and 30.8% after stimulation)(139).

Simultaneous detection of pepsinogens and *H. pylori* antibodies have been suggested by Japanese investigators, this approach is known also as the ABC(D)-method (119, 140). Individuals are classified as group „A" if pepsinogen levels are normal and *H. pylori* antibodies absent, group „B" if pepsinogen levels are normal and *H. pylori* antibodies present, group „C" if pepsinogen levels are decreased and *H. pylori* antibodies present, group „D" if pepsinogen levels are decreased and *H. pylori* antibodies absent. Based on the results individual risk assessment can be optimised and endoscopic surveillance individually taylored (141, 142). Additionally to PgI, PgII and *H. pylori* antibodies, assessment of <g17 can also be included in the test-panel for a higher diagnostic yield (116, 133).

Several studies have demonstrated that decreased pepsinogen levels (alone or in combination with anti-*H. pylori* antibodies and/or G-17) are predictive for gastric cancer development.In the Hisayama study (143) 2,446 subjects aged 40 years and above were followed prospectively for 14 years. The hazard ratio (HR) for developing cancer was substantially higher for the group with decreased pepsinogen levels than in individuals with normal test results (HR 4.6, 95% CI: 2.4 - 8.6 for men;HR 5.8, 95% CI: 2.0 - 17.1 for women). Similarly, 5,706 male employees aged 40-60 years were followed for 10 years in the Wakayama City study (144). The HR for gastric cancer development was 5.2 (95% CI: 2.8-9.5) for patients with significantly decreased Pg levels. Watabe at al. (140) followed 6,983 individuals for the duration of 4.7 years. Hazard ratios for gastric cancer incidence were 6.0 (95% CI: 2.4-14.5) in the group of decreased pepsinogen and positive anti-*H. pylori* antibodies (group C according to the „ABC(D)" classification), and 8.2 (95% CI: 3.2-21.5) in the group of decreased pepsinogen but negative anti-*H. pylori* antibodies (group D). The study demonstrated that a decrease of anti-*H. pylori* antibodies during advanced stages of atrophy is related to an even higher risk. In contrast to these results, a long-term study in a high-risk area of China, showed a higher risk in individuals with decreased pepsinogens and positive anti-*H. pylori* antibodies. This group demonstrated an increase in the relative risk (RR) of 27.5 (95% CI: 3.4-225.4), while in the group with decreased pepsinigens and negative anti-*H. pylori* antibodies the RR was 23.2 (95% CI: 2.1-260.9) (145). Recently, an initial report from a case-control study in Russia indicated a HR for gastric cancer development of 4.31 (95% CI: 1.5 -12.5) for PgI/II < 3.0(146). Data from Japan has demonstrated that a serological screening for pepsinogens with further referral for upper endoscopy or photofluoroghaphy if the test is positive, has been capable to decrease gastric cancer deaths by 76% (in individuals screened 1 year before the diagnosis) or by 62% (in individuals screened within 2 years) (147).

A large cohort study in a population from Portugal demonstrated the feasibibility of pepsinogen testing approach even in a Western population (148). A total of 13,118 individuals have been followed for five years. Of the 446 individuals (3.4%) with decreased pepsinogen levels, 274 underwent upper gastrointestinal endoscopy; six cancer cases have been detected representing one cancer per 2,200 tests or one incident case per 74 positive tests (148).

Different cut-off values to for determining decrease in the pepsinogen levels have been used in many studies (149), and different test-systems are traditionally used in Asia and Europe. Therefore, the results in absolute values cannot be directly compared between the different studies(150). Based on the above, the current guidelines emphasise the need for regionally validated test-systems (151).

5.2. Cancer autoantibodies

Autoantibodies against tumor-associated antigens have been identified in several cancer types (152-154).Due to their specificity and stability in the serum, they represent attractive targets for the development of noninvasive serological tests for the early detection of cancer (155). However, the frequency of antibodies against particular tumor associated antigens is rather low, typically ranging 1-15%, therefore an approach of panel-testing is frequently been used to explore cancer-specific antibodies (155).Recently, a 45 cancer-associated autoantibody signature was identified able to discriminate gastric cancer from healthy controls with 58.7% sensitivity and 89.7% specificityby using a T7 phage-displayed SEREX approach combined with the phage-displayed antigen microarray technology and a novel strategy for the analysis of microarray data(155). There is a potential to increase further the sensitivity of this test.

5.3. Circulating cancer cells

Identification of circulating tumour cells (CTCs) has been related to the presence of a systemic disease and appearance of peripheral metastasis (156). Detection of CTCs is suggested useful for estimation of the individual prognosis and for monitoring several cancer types, including breast, lung, prostate, skin, colon and gastrointestinal cancers (157-159).

A larger number of CTCs has been found in metastatic gastric cancer than non-metastatic disease with the identification of two or more CTCs being an indicator for more advanced stage disease as well as peritoneal dissemination (160). In a recent study by using a telomerase-specific viral agent to detect CTCs in peripheral blood of gastric cancer patients a significant relationship between the number of CTCs and the prognosis of the disease was revealed. However, not all the CTCs might have equal metastatic potential since here, recurrence of early stage disease was not identified even in the presence of CTCs (159). However, the direct detection of gastric cancer derived CTCs is difficult and requires high methodological effort. Therefore, there have been approaches to use RNA products of these cells as surrogate (94, 161, 162).

An alternative to CTCs is the detection of circulating nucleic acid shed from necrotic or apoptotic

cells (163). Using mutation or methylation specific assays it is possible to quantify the fraction of DNA originating directly from tumour cells as well as the total levels of circulating nucleic acid (164, 165). Quantification of the level of CNA has been demonstrated to be a sensitive early marker of disease response to chemotherapeutic and surgical treatments (164, 166).

Several studies have demonstrated elevated levels of circulating nucleic acid in gastric cancer though as yet the clinical relevance of this remains unclear (167, 168).

6. VOLATILE ORGANIC COMPONENTS IN BREATH

The main constituents of human breath are nitrogen, oxygen, carbon dioxide, water vapour and inert gases. In addition, thousands of volatile organic compounds (VOCs) are exhaled at very low concentrations (estimated as parts per trillion (ppt) or parts per billion (ppb) by volume of the exhaled breath) (169, 170). While part of the substances is of endogenous origin and could be characteristic for metabolic processes in the human body (including cancer), others are exogenic, i.e. passing through the human body (169, 171).

Initial results on the potential applications of volatile marker tests for detection of several cancer types have been published, e.g. for lung, breast, colorectal and prostate cancers (172, 173),and hepatocellular carcinoma (174).

A recently published study addressed the potential of volatile markers to diagnose gastric cancer (175). By using novel cross-reactive, highly sensitive gas sensor allowing to identify and separate VOC patterns, the obtained results demonstrated 89% sensitivity and 90% specificity to differentiate gastric cancer cases from non-malignant conditions after cross-validation, irrespective of important confounding factors such as tobacco or alcohol consumption and *H. pylori* infection (176). Further validation and reproducibility studies are required, including different populations.

7. MOLECULAR DIAGNOSTICS

7.1. MicroRNA

MicroRNAs (miRNA) are short RNA molecules of approximately 22 nucleotides that are involved in posttranslational regulation of gene expression (177, 178). Dependent on the sequence homology of miRNAs and targeted RNA the effect may vary from partial inhibition of the mRNA translation to cytoplasmatic degradation of the mRNA transcripts (179, 180). Thus, each of currently known human miRNAs may control hundreds of mRNA targets and so be involved basically in every cellular process which becomes obviously deregulated during the carcinogenesis. For the detailed introduction into the miRNA biogenesis we refer to recently published excellent reviews (181-183). In this section, we will briefly introduce the most important aspects of miRNA research in regard to gastric cancer development and biomarker research.

Following the pivotal work of Lu *et al.* demonstrating differential miRNA expression across various tumours by using 217 miRNAs (184), an increasing number of studies have clearly and consistently shown differential expression of miRNAs in gastric cancer compared to normal tissues. At present, Ueda and colleagues have performed the largest study on gastric cancer tumours (185). Using a respectable number of gastric cancer tissues paired with non-tumour samples the authors identified 22 up- and 13 downregulated miRNAs. Additionally, using the pattern of the 19 most significantly deregulated miRNAs it was possible to discriminate gastric tumours according to their histological type. In particular, cluster analyses revealed miR-105, -100, -125b, -199b, -99a, 143, -145 and -133a upregulated in diffuse type gastric cancer, while miR-373-3p, -498, -202-3 and -494 were upregulated in intestinal type lesions. Some miRNAs were related to the stage of disease, and, most importantly, let-7g, miR-214 and -433 were identified as independent prognostic biomarkers predictive for overall survival in gastric cancer patients (185).

There is increasing evidence that certain miRNA patterns are also associated with premalignant stages or even risk conditions like *H. pylori* driven inflammation (186, 187). Interestingly, *H. pylori* eradication can result in at least partial normalization of the deregulated miRNAs, further underlining the clinical importance of miRNAs in the initiation and progression of gastric cancer.

Furthermore, an increasing number of studies provided substantial evidence for applicability of miRNAs as non-invasive biomarkers for gastric cancer (188, 189). MiRNAs are easily and reproducibly detectable in various body specimens including blood(188, 189), gastric fluids (190, 191), faeces (192, 193), saliva (194) and others (195). A remarkable stability of miRNAs from degradation in body fluids is perhaps one of the most exciting characteristic of miRNAs which has been linked to the ability to build protein complexes and be sequestered in exosomes (196, 197). Although the mechanism of miRNA extravasation is not completely understood, it is believed that either active release of exosomes or passive extravasation of the protein complexes during apoptosis or cell death are the main source of circulating miRNAs (196-198). This is further supported by the fact that expression pattern of circulating miRNAs change dramatically following surgical resection of the tumour; however, exosome release is not a tumour-specific event but a rather ubiquitous process, and it becomes clear that not only direct tumour-related release, but also indirect changes in miRNA expression (stroma- or immune cells) may be a cause of measurable deregulation of circulation miRNAs. After the publication of the first data, several groups have confirmed the differential expression of certain miRNAs in the blood of cancer patients, partially using high-throughput techniques (199-201). So far, there has been only minor attention paid to the association between *H. pylori* and gastric cancer associated circulating miRNAs. It has been

shown, that levels of miRNA expression in sera were highly correlated with *H. pylori* status both in gastric cancer patients and *H. pylori* infected controls, and particularly miR-223 was present at a significantly higher level in *H. pylori* infected individuals compared to those without the infection (199).

Another elegant approach is the use of gastric juice for miRNA expression analysis. Guo *et al.* assessed miR-421, -129, -21 and -106b expression in the gastric juice of patients with mild superficial gastritis, atrophic gastritis, gastric ulcer or gastric cancer. Although all tested miRNAs we concluded to be promising for the screening of gastric cancer, prospective validation in independent patients cohort is still needed (190, 191, 202).

Mutation or specific variations in the DNA sequence of miRNA genes may be associated with alterations in miRNA processing, if the base pair changes are located in precursor sequence, or it may influence the functional interaction of miRNA with a target molecule, if located in a miRNA coding sequence. Two miRNA-SNPs have been in primary focus of several studies, rs2910164 G>C of the pre-miR-146a and rs11614913 of pre-miR-196a-2 (203-205). Although the results are not homogenous, it seems that at least in Asian gastric cancers, these SNPs may be associated with susceptibility for atrophic gastritis or an increased risk for gastric cancer. Large unbiased genome-wide multicentre studies will be needed to estimate the exact meaning of those changes.

In similar fashion as coding mRNA, transcription of miRNA genes is regulated by epigenetic status of the promoter regions. DNA methylation together with histone modifications are associated with differential miRNA expression by regulation of the accessibility of certain transcription factors. Ando *et al.* observed an up to 13-fold increase in the methylation level of miR-124a in gastric biopsy samples from patients with *H. pylori* infection(206). Several other miRNAs miR-137, miR-34b or -129-3p have been also suggested, but systematic quantitative methylation analyses are still missing(207, 208).

Taken together, miRNA research has been truly a fast break event. At present, the unique features of miRNA with a rapidly increasing amount of data stands next to a lack of understanding in regard of multistep process of carcinogenesis, and unanswered methodological challenges need to be solved prior the broad clinical implementation of miRNAs as biomarkers. As for other biological markers assessed by high throughput techniques, biostatistical evaluation of miRNA patterns facilitate the identification of signature that can be uniquely attributed to certain diseases. It has been shown recently, that the differential expression of miRNA signatures can be assessed for the distinction of gastric and oesophageal adenocarcinoma (209). These approaches can support a better understanding of the different pathobiological pathways leading to cancer of the oesophagogastric junction of either gastric or oesophageal origin.

7.2. Epigenetic changes

Epigenetic mechanisms refer to different modifications of the chromatin structure that affect gene expression without altering the primary DNA sequence. The two major epigenetic mechanisms that lead to activation or silencing of the gene function are methylation and histone modification, which are related to the development of different cancers, including gastric cancer (210).

Global hypomethylation and promoter hypermethylation are common features in various gastrointestinal malignancies. The first feature refers to the loss of DNA methylation and is linked with genomic instability and tumor formation, while promotor hypermethylation causes transcriptional silencing of tumor suppressor genes and may affect important molecular pathways (211).

Methylation of CpG islands has been assessed in promoters of different genes related to gastric carcinogenesis. A growing number of genes related to cell cycle, apoptosis, tumour invasion, cell adhesion, cell signaling, and transcription have been shown to be silenced by hypermethylation in gastric cancer. Genes encoding *CDH1* (212), *FOXD3* (213), *RUNX3* (214), *TPEF* (215), and other well-known tumor suppressor genes have been shown to be affected by methylation. As mentioned above, recent studies on small non-coding miRNAs have also revealed modifications of epigenetic regulation that occur in gastric cancer (182). The genes that are effected by methylation in gastric cancer are extensively discussed in several reviews (216-218).

Studies on *H. pylori* and *Epstein-Barr virus* (EBV) infection show that the carcinogenic effect of these pathogens may be reinforced by inducing methylation changes in the gastric mucosa (214, 219). Interestingly, inflammation induced by *H. pylori* infection was critical for methylation induction of promoters containing CpG islands through the release of reactive oxygen species and nitric oxide and by activation of the DNA methyltransferase (220). Furthermore, epigenetic changes in gastric cancer occur not only at the stage of malignancy, but also at early stages of cancer development including atrophic gastritis and IM (221). There are convincing data showing that methylation profiles are different for intestinal and diffuse type gastric cancer (222).

Pyrosequencing and other technological developments enabled researchers to look not only at methylation status in individual oncogenes, but also allowed to assess global methylation patterns in gastrointestinal malignancies. Global demethylation of the tumour cell genome in gastric cancer occurs in parallel to abnormal hypermethylation of tumour suppressor genes (223). Data on global-methylation status in gastric cancer suggest that this could be used as a marker to detect metastasis and may reflect the malignant potential of gastric cancer(224). Further studies on epigenetic changes indicate that global methylation occurs in *H. pylori* induced gastritis representing an early event in gastric carcinogenesis (225).

As the methylation status in the tumour tissue is resembled by patterns retrieved from serum samples, methylation status has the potential to become a non-invasive marker, which could be used for early diagnostics of gastric cancer and a novel target for cancer prevention(226). Furthermore, different studies showed that epigenetic changes are predictors for response to chemotherapy and patient survival and thereby might influence the decision making process in the treatment of these patients (217). Up to date, methylation based non-invasive testing is not yet available for diagnostic, prognostic or treatment-related decision making in the clinical setting, but ongoing work in the field may reveal significant benefits for the patients in the near future.

7.3. Genetic alterations

Genetic variation influences individual susceptibility to different malignancies, including gastric cancer (227). Significant advances in molecular biology, next generation sequencing and bio-banking activities enabled researchers to examine different genetic entities in relation to numerous human diseases. Genetic predisposition to sporadic gastric cancer remains largely not understood (228). The only genetic test which can be applied in today's clinical practice is screening for *e-cadherin* (*CDH1*) mutations in familial cancer cases, however, this approach is applied in very few specialised centers (229).

The most common type of genetic variation in the human genome is single nucleotide polymorphisms (SNPs). A currently accepted hypothesis suggests that individuals with a pro-inflammatory genetic profile are more vulnerable to *H. pylori* infection related damage and gastric cancer development. Chronic inflammation is the major event in the model of gastric carcinogenesis, which has been demonstrated in human and animal studies (230, 231). Based on this paradigm, genes responsible for inflammation and *H. pylori* recognition became a major interest of different gastric cancer research groups. In the year 2000, the association between a pro-inflammatory *interleukin-1β* (*IL1B*) gene polymorphism and an increased risk of *H. pylori*-induced gastric cancer was reported (232). This paper was followed by numerous case-controls studies on genes encoding inflammatory cytokines, Toll-like receptors (TLRs), Nucleotide-binding oligomerisation domain (NOD) receptors and other genes involved in *H. pylori* induced carcinogenesis (233). Initial studies have reported a significant effect of certain cytokine gene alterations for the risk of gastric cancer development (232), while data from more recent studies have showed marginal or no associations (234-237). Interestingly, some research groups described a relation between cytokine SNPs and progression of mucosal inflammation and atrophy(238), while genetic variations of TLR receptors might be linked with reduced risk of *H. pylori* induced diseases in the stomach(239). Polymorphisms of *IL1B* and *interleukin-1 receptor antagonist* gene (*IL1RN*) are best studied in the context of gastric cancer development. Several meta-analyses have been published summarizing the results of the smaller case-control studies on *IL1B* and *IL1RN* SNPs in relation to gastric cancer risks (240-243). Based on the conclusions of these meta-analyses, the overall impact of *IL1B* and *IL1RN* genetic alterations for gastric cancer development appears to be marginal and questions the applicability of these SNPs as potential screening biomarkers.

The major shortcoming of small hypothesis driven case-control genetic association studies discussed above is related to the small number of individuals within the groups, which are poorly stratified according to individual variables (histological subtype, *H. pylori* status, anatomical site of the tumor, etc.). New high-throughput technologies enabled researchers to perform large genotyping association studies. Genome wide association studies (GWAS) on gastric cancer have identified several interesting candidate genes that could serve as non-invasive markers for early detection. *PSCA*, *PLCE1* and *MUC1* gene polymorphisms have been linked with gastric cancer risk in recent GWAS studies (244, 245). It is worth noticing that for some of the SNPs revealed in gastric cancer GWAS studies have also implicated in the formation of premalignant gastric conditions (246). GWAS studies on gastric cancer may serve not only for identification of genetic susceptibility loci, but may also reveal previously unknown pathways in gastric carcinogenesis.

To date, however, none of genetic alterations can be used in daily clinical practice for stratification of risk of sporadic gastric adenocarcinoma in an individual patient (151).Guidelines on the management of precancerous gastric conditions and lesions (MAPS) draw a similar conclusion stating that despite numerous studies on host genetic variations, no clinical recommendations can be made for targeted management based on these factors with regard to diagnosis and surveillance (49, 247).

8. BIOSTATISTICAL ASSESSMENT

The development of tissue-based microarrays and chromatin immuno-precipitation (ChIP-) analysis opened the gate to a system-wide evaluation of differential gene-expression related to a variety of diseases (248). The application of biostatistical algorithms enables the structured assessment of the tremendous amount of data that is delivered by array-based techniques. Principal component analyses and hierarchical clustering enable functional classification of the identified gene expression networks and even individual genes and related signalling pathways (249). By this approach putative target genes with prognostic or therapeutic potential can be identified as well as further interacting partners (250, 251). These target genes don't necessarily have to be deregulated below or above a certain threshold (252), comparison to „normal" tissue or between different entities of malignant diseases defines central node-genes like transcription factors or cell cycle regulators (253). This approach can be applied for the definition of pathogenetic patterns of non-malignant diseases (254, 255)as well for specific oncological diseases, e.g. neuroendocrine tumours, hepatocellular carcinoma, breast and prostate cancer (256-259).

Computational visualisation of gene-gene-interaction networks and the related gene-clusters enables also a topological assessment of gene-coexpression as well as the integration of multiple datasets for a comprehensive

analysis (251, 260). The results are based on features of the ENSEMBL database and classified by "gene-ontology" terms: a) biological process, b) molecular function, c) cellular component (261). The algorithms in use are under permanent development for further optimisation of the gain of information. By genome-wide association studies (GWAS) of gene aberrations or specific expression profiles, classifiers for an individual prognostic assessment, a treatment susceptibility profile or as diagnostic marker panel can be generated from analyses on human tissue or derived cell lines (244, 262, 263).

A further basic approach is the classification or clustering of copy number aberrations, gene amplifications or deletion. Patients can be stratified in distinct subgroups according to their genetic tumour profile, that can even be associated with clinicopathological characteristics like tumour location, TNM stage including depth of invasion and lymph node involvement, or expression of certain receptor subtypes like Her2 (264-266). However, it is still questioned if the detected alterations define distinct subtypes of gastric adenocarcinomas with common genetic profiles, or if an individual "evolution" of certain tumour subclones is responsible (266).

Recently, there has been a focus on genes that are targetable by medical treatment (EGFR, MET, FGFR, HER2) (267, 268). SNPs or copy number aberrations in the respective genes might enable the prediction of the prognostic outcome after neoadjuvant or even palliative therapy. For validation of the identified gene signature and the biological relevance, assessment on protein level by immunohistochemistry or protein expression arrays is useful (265). By "array comparative genomic hybridisation" different levels of gene expression can be taken into account to demonstrate that basic genomic alterations have an actual influence on the related protein profile (269, 270). In a study on tissue from oesophageal adenocarcinomas a genome-wide SNP array was applied in case of differential gene regulation in the microarray data (271). The thereby identified target genes have then been validated by either PCR or FISH analysis. Traditional immunohistochemistry was used for definitive validation in a different study population, including the clinical data for prognostic relevance of the signature by Kaplan-Meier analysis (271).

It is not mandatory to generate „de novo" data for the initial biostatistical evaluation. The computational algorithms can also be applied on publicly available array datasets. Data published in 2006 demonstrated new molecular targets in gastric cancer, derived from formerly published datasets. However, the validation has then been performed in a prospective study population (272). This new field of computational *"in silico"* analysis of data generated by using available array platforms enable the identification of target signatures that can be correlated with any tumour specific feature, like the degree of differentiation, general tumour stage, or survival and outcome after surgical treatment (264, 273). The underlying computational algorithms can be modified according to the clinical question that should be answered. To enable the implementation of the identified classifiers in clinical practise, it is of high importance to keep the number of involved genes and their products as low as possible (274). Thus, a prognostically relevant signature containing only two genes has been identified for oesophageal adenocarcinomas indicating the clinical response and outcome after radio-chemotherapy (275). Others demonstrated a four-genes signature significantly associated with five-years survival as independent prognostic factor in multivariate analysis(276).

By the analysis of genes that regulate stromal invasion of tumour cells, the hierarchical cluster analysis revealed a stepwise differential regulation of genes that are in involved in the transition from metaplastic Barrett's epithelium to invasive adenocarcinoma of the distal oesophagus and the gastro-oesophageal junction(277). The related genes had mainly immune-modulatory function, regulating the cytokine-cytokine-receptor interaction and TGF-β-dependent signalling pathways. This could be shown for different adenocarcinomas of the gastrointestinal tract including gastric cancer (277).

It has been demonstrated that the related gene expression signatures that lead to activation of major oncogenic pathways include regulators of stem cell proliferation and NFκB-, Wnt/β-catenin-related signalling pathways, and are deregulated in more than 70% of the analysed cancer samples (278). A major task is the identification of mechanisms that make the carcinoma prone to local invasion and metastatic spread (279). However, even in the same organ of origin – e.g. the stomach - the involved regulatory processes show different patterns depending from the tumour localisation. Respective differences could be identified for proximal versus distal gastric cancer of the intestinal type, especially, when compared with diffuse type neoplasias (280, 281). Gastric cancer subtypes, identified by their gene expression signature as well as by the patterns of copy number aberrations and epigenetic changes, have been demonstrated to present distinct clinical response to treatment (282). Lei *et al.* presented data on a stratification into three different gastric cancer subtypes showing not only specific pathobiological characteristics but also remarkable differences concerning the response to treatment with either 5-fluorouracil or compounds targeting the PI3K-Akt-mTOR axis (282). These data facilitate the design of clinical trials and the use of small molecule targeting therapeutic agents (283).

Besides genetic alterations there have been numerous attempts to analyse directly the changes on protein level to identify protein signatures that are related to TNM stage and the degree of tumour differentiation (284). This "Proteomics" approach was also successfully used for prediction of response to neoadjuvant therapy or even to specific agents like MET-pathway inhibitors (285, 286). The data generated by high-throughput techniques like matrix-assisted laser desorption/ionisation (MALDI) imaging can be entered in similar computational algorithms that enable hierarchical clustering and general principal component analysis (287), and the related protein

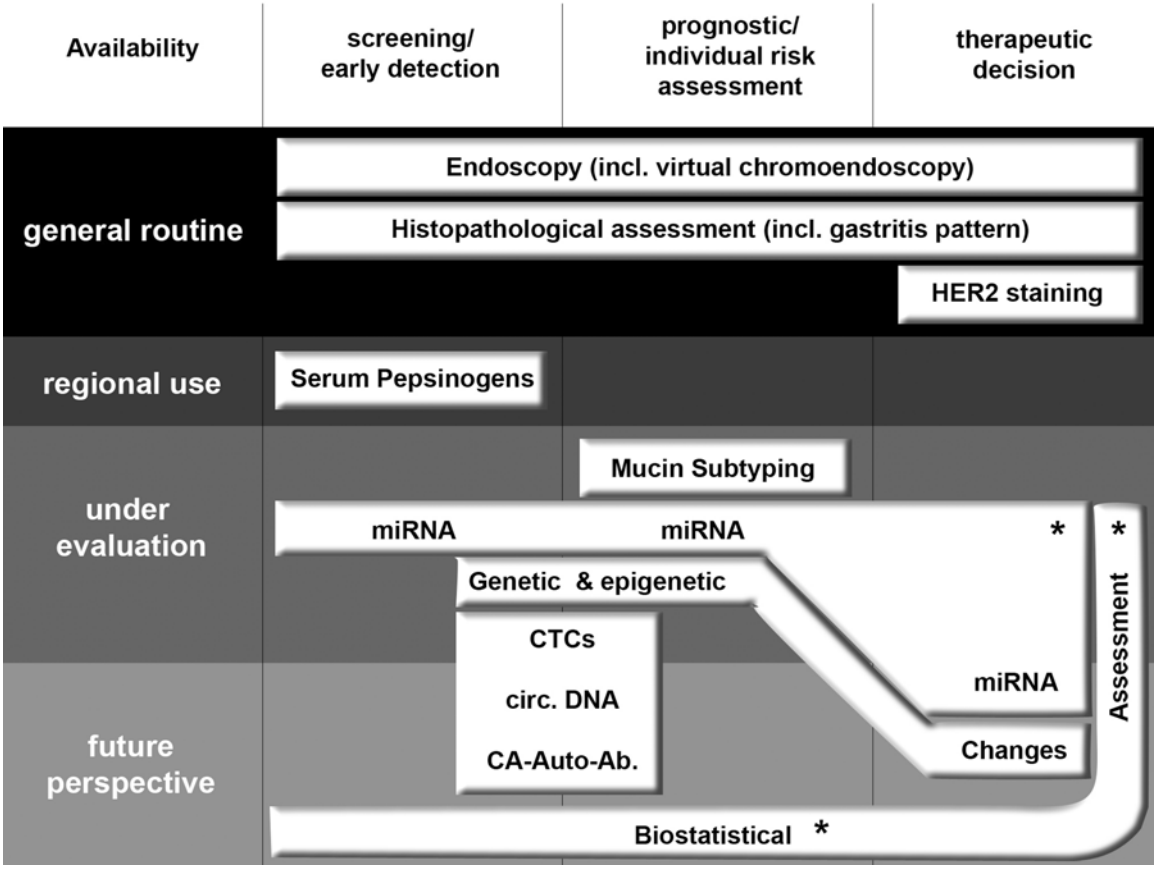

Figure 3. Overview on diagnostic tools for gastric cancer assessment. The image gives an overview on the techniques that are either established or under development for gastric cancer diagnostics. These approaches can be either applied for basic diagnostic assessment including population-based screening, for primary staging of the disease to define the optimal treatment strategy, or for prognostic assessment of manifest disease. Indicated are also regional differences concerning the availability of certain techniques. Techniques marked with an asterix (*), e.g. miRNA and biostatistical assessment, are already in use in a broad perspective, but can still not be regarded for application in clinical routine.

signatures are also capable to differentiate between different tumour types, organ sites or even biological behaviour of metastases (288). An important step in this field is the identification of blood-based parameters. Some groups reported tumour-specific serum biomarkers, but up to today the sensitivity and specificity of these marker panels is far too low to be transferred into clinical practise (96, 289).

9. VIEW TO THE FUTURE AND CONCLUSION

Although the incidence of gastric cancer is globally declining, the disease will still represent a major healthcare issue during the decades to come; therefore, identification of individuals at risk and detection of the disease at early stage remains a big challenge. The exponentially rising costs for targeted treatment modalities in case of advanced stage disease require strong and consistent predictors for treatment response to guide the therapeutic decision.

Biomarker testing with pepsinogens for identifying individuals at increased risk for cancer development is considered by the guidelines in the East and West (49, 151, 290). Although there have been recent significant advances concerning the techniques applied that opened the gates to a variety of blood or tissue based investigations, none of these is at the step to a routine application in a clinical setting yet.

At the moment there are no perfect non-invasive screening tools for gastric cancer available. The most extensive studied of the available tests are serum pepsinogens; however, even there additional data are necessary before these can be recommended for organised screening programs, especially in non-Asian countries.

New and exciting developments are currently under investigation, these include miRNA signatures, cancer autoantibody panels, volatile components in exhaled breath, and other (Figure 3). However, there is still way to go before they could be available to the practice; in addition cost-effectiveness will have to be addressed before such tests could be implemented to population-based screening programs for gastric cancer.

10. ACKNOWLEDGEMENTS

We thank Dr. Jochen Weigt, Otto-v.-Guericke University of Magdeburg, Germany, for the endoscopic images (Figure 1).

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Key Words: Gastric cancer, endoscopic imaging, Her2, pepsinogens, miRNA, array comparative genomic hybridisation, mucins, Review

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