DNA METHYLATION BIOMARKERS IN CANCER DIAGNOSTICS

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Abstract

Cancer development and progression are characterized by intricate genetic and epigenetic alternations, with DNA methylation playing a crucial role in this transformative process. Recent advancements in DNA methylation mapping technologies have significantly contributed to the identification of novel cancer biomarkers. We provide a short overview of Conformité Européenne-marked in vitro diagnostic tests designed to detect DNA methylation changes in oncology practice.

Keywords: DNA methylation, cancer, biomarker

INTRODUCTION

Cancer development is intricately linked to the gradual genetic changes that involve mutations in oncogenes, tumor suppressor genes (caretaker and gatekeeper genes), and chromosomal abnormalities. Beyond this genetic landscape, it has been confirmed that epigenetic modifications also play a substantial role in initiating and advancing cancer. These modifications entail heritable alterations to DNA without any change in its nucleotide sequence.

The most extensively studied epigenetic modification within the human genome is DNA methylation. This process involves the covalent addition of a methyl group to the 5-carbon of the cytosine ring in CpG sequences, ultimately yielding 5-methylcytosine. The methyl group transfer occurs through a reaction catalyzed by DNA methyltransferases, utilizing S-adenosylmethionine as the methyl group donor. In the context of tumor cells, DNA methylation undergoes a distinctive redistribution, manifesting as a combination of global genomic hypomethylation and localized CpG island hypermethylation. Subsequently, the hypermethylation of promoter regions associated with tumor suppressor genes or genes governing cell cycle control, apoptosis, and drug sensitivity leads to transcriptional silencing (1). This epigenetic modulation contributes to the inactivation of critical pathways, promoting uncontrolled cell proliferation and resistance to therapeutic interventions that are often characteristic of cancer development and progression.

The early occurrence of epigenetic modifications in cancer pathogenesis suggests their potential as promising biomarkers for screening and early detection. The further potential lies also in their reversibility, offering a unique possibility for the development of innovative treatment options. Moreover, the recent advancements in DNA methylation mapping

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technologies have significantly increased opportunities for epigenetic research. This progress has facilitated the identification of novel epigenetic biomarkers with potential practical application in clinical care (2). However, before the tests based on these biomarkers can be integrated into clinical practice in the European Union (EU), they must undergo clinical validation to ensure their results align with the In Vitro Diagnostic (IVD) Device Regulation.

In the following section, we provide an overview of IVD oncology tests designed to detect DNA methylation changes that obtained Conformité Européenne (CE) marking.

DNA METHYLATION CANCER BIOMARKERS APPROVED FOR CLINICAL USE IN THE EUROPEAN UNION

Globally, over thirty DNA methylation-based assays designed to assist in clinical decision-making in various types of cancer have been introduced to the market (3,4). Not all of them have fulfilled the requirements of EU IVD regulation. The tests approved for use in the EU are summarized in **Table 1**.

Currently, there are three IVD tests available to help with the diagnosis of lung cancer. Epi proLung BL Reflex Assay, manufactured by Epigenomics AG, Berlin, Germany, detects the methylation of the *SHOX2* gene in bronchial aspirates from patients at an increased risk of the disease (5). The manufacturer has adapted the test for use in plasma samples, resulting in Epi proLung, which enables the discrimination between lung cancer and non-cancerous diseases based on a combined methylation analysis of *SHOX2* and *PTGER4* genes (6). A novel blood-based test, named PulmoSeek from AnchorDx Medical Company, Guangzhou, China, detects methylation in a panel of 100 pre-selected lung cancer-specific methylation regions and can differentiate malignant from benign pulmonary nodules (7).

Another DNA methylation-based test developed by Epigenomics AG is intended for use in screening for colorectal cancer. The Epi proColon test uses peripheral blood samples to analyze the methylation status of the *SEPT9* gene (8,9). The test has also been adapted for the early detection of hepatocellular carcinoma under the name HCCBloodTest (10). Completely non-invasive is the EarlyTect Colon Cancer test, manufactured by Genomictree, Daejeon, South Korea, that targets *SDC2* gene methylation in DNA extracted from stool (11).

Three CE-IVD tests (therascreen *MGMT* Pyro Kit, Qiagen, Hilden, Germany; Human *MGMT* Gene Methylation Detection Kit, Xiamen Spacegen, Xiamen, China; and *MGMT* Methylation Detection Kit, EntroGen, Los Angeles, CA, USA) were developed to predict the effectiveness of temozolomide therapy in the most common malignant brain tumor in adults, glioblastoma (12). All the tests detect the methylation of distinct CpG sites of the *MGMT* gene in the DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue samples.

Several methylation-based tests are also available for early diagnosis of cervical cancer or advanced cervical intraepithelial neoplasia, a premalignant lesion that precedes cervical cancer. For this purpose, Qiagen offers a test named QIAsure that analyzes the methylation status of the *FAM19A4* gene and microRNA hsa-mir124-2 in cervical or vaginal specimens (13). GynTect (oncgnostic, Jena, Germany) targets the methylation of the six-gene panel (*ASTN1*, *DLX1*, *ITGA4*, *RXFP3*, *SOX17*, and *ZNF671*) in the DNA extracted from cervical smears of HPV-positive women (14). The iStat Biomedical (New Taipei City, Taiwan) developed two tests for the detection of either cervical or oral cancer in the DNA from cells collected by the scraping: *PAX1* DNA Detection Kit and *ZNF582* DNA Detection Kit (15).

Another CE-IVD test from Qiagen, the therascreen *PITX2* RGQ PCR Kit, is intended for predicting the outcomes after anthracycline based chemotherapy in patients with high risk breast cancer. The test detects the methylation of three CpG sites within the *PITX2* gene in the DNA extracted from FFPE tissue (16).

To help with bladder cancer diagnostics and surveillance, two non-invasive tests are available based on urine DNA methylation detection. The UriFind Bladder Cancer Detection Kit from AnchorDx detects *ONECUT2* and *VIM* methylation (17). The Bladder EpiCheck (Nucleix, Rehovot, Israel) is a post-treatment monitoring test that targets methylation changes in 15 proprietary biomarkers and is intended for recurrence detection (18).

The EsoGuard assay developed by Lucid Diagnostics (Gilbert, AZ, USA) can assist with detecting the precursor condition of esophageal cancer called Barrett's Esophagus. The minimally invasive test targets the hypermethylation of the *CCNA1* and *VIM* genes in esophageal brush cells (19).

The microarray DNA methylation signatures detected in fresh frozen or FFPE tissue are the base for the EPICUP test developed by Grupo Ferrer Internacional SA (Barcelona, Spain). This primary tumor type classifier presents an invaluable tool for improving the diagnosis of cancers of an unknown primary and the following choice of treatment (20).

OverC Multi-cancer Detection Blood Test (Burning Rock Biotech, Guangzhou, China) was designed to aid in the early detection of multiple cancers, including esophageal, liver, lung, ovarian, and pancreatic cancers (21). The test is intended for liquid biopsies and targets the circulating tumor DNA methylation.

The IVD tests listed above have received approval for use in the EU. However, there is a potential for the additional kits approved in the USA by the Food and Drug Administration to extend their reach to the European market.

CONCLUSION

The absence of definitive biomarkers in oncology poses a significant challenge in cancer research. The dynamic and responsive nature of epigenetic modifications, such as DNA methylation, makes them an attractive target and underlines their potential diagnostic and prognostic values. As indicated by the number of previously mentioned tests, the integration of novel methylation biomarkers into clinical practice has clearly begun and holds the promise to improve cancer diagnostic and treatment strategies in the foreseeable future.

Gene abbreviations

ASTN1	*Astrotactin 1*
CCNA1	*Cyclin A1*
DLX1	*Distal-Less Homeobox 1*
FAM19A4	*TAFA Chemokine Like Family Member 4*
ITGA4	*Integrin Subunit Alpha 4*
MGMT	*O-6-Methylguanine-DNA Methyltransferase*
ONECUT2	*One Cut Homeobox 2*
PAX1	*Paired Box 1*
PITX2	*Paired Like Homeodomain 2*
PTGER4	*Prostaglandin E Receptor 4*
RXFP3	*Relaxin Family Peptide Receptor 3*
SDC2	*Syndecan 2*
SEPT9	*Septin 9*
SHOX2	*Short Stature Homeobox 2*
SOX17	*Sex Determining Region Y-Box Transcription Factor 17*
VIM	*Vimentin*
ZNF582	*Zinc Finger Protein 582*
ZNF671	*Zinc Finger Protein 671*

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Table 1 Commercially available DNA methylation-based tests in oncology with CE-IVD certification

Test name	Methylation biomarker	Biological sample	Company	EU certification
Lung cancer				
Epi proLung BL Reflex Assay	*SHOX2*	Bronchial aspirate	Epigenomics AG, Germany	2010
Epi proLung	*SHOX2*, *PTGER4*	Plasma	Epigenomics AG, Germany	2017
PulmoSeek	100 biomarkers	Plasma	AnchorDx, Hong Kong	2022
Colorectal cancer				
Epi proColon	*SEPT9*	Plasma	Epigenomics AG, Germany	2011
EarlyTect Colon Cancer	*SDC2*	Stool	Genomictree, South Korea	2017
Glioblastoma				
therascreen MGMT Pyro Kit	*MGMT*	Tumor tissue	Qiagen, Germany	2012
Human MGMT Gene Methylation Detection Kit	*MGMT*	Tumor tissue	Xiamen Spacegen, China	2016
MGMT Methylation Detection Kit	*MGMT*	Tumor tissue	EntroGen, USA	2018
Cancers of Unknown Primary				
EPICUP	multiply CpG sites	Tumor tissue	Ferrer Internacional, Spain	2015
Cervical cancer				
QIAsure	*FAM19A4*/miR124-2	Cervical/vaginal specimen	Qiagen, Germany	2016
PAX1 DNA Detection Kit	*PAX1*	Cervical/oral scrapes	iStat Biomedical, Taiwan	2016
GynTect	*ASTN1*, *DLX1*, *ITGA4*, *RXFP3*, *SOX17*, *ZNF671*	Cervical smear	oncgnostic, Germany	2019
Oral cancer				
ZNF582 DNA Detection Kit	*ZNF582*	Cervical/oral scrapes	iStat Biomedical, Taiwan	2016
Bladder cancer				
Bladder EpiCheck	15 proprietary markers	Urine	Nucleix, Israel	2017
UriFind Bladder Cancer Detection Kit	*ONECUT2*, *VIM*	Urine	AnchorDx, Hong Kong	2020
Hepatocellular carcinoma				
HCCBloodTest	*SEPT9*	Plasma	Epigenomics AG, Germany	2018
Esophageal precancer				
EsoGuard	*CCNA1*, *VIM*	Brush cells	Lucid Diagnostics, USA	2021
Breast cancer				
therascreen PITX2 RGQ PCR Kit	*PITX2*	Tumor tissue	Qiagen, Germany	2018
Multiple cancer types				
OverC Multi-cancer Detection Blood Test	ctDNA markers	Plasma	Burning Rock Biotech, China	2022

The role of selected matrix metalloproteinases in the pathogenesis of gliomas

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Abstract

The progression of tumor formation is a multifactorial process that involves changes at different levels. Within this intricate molecular and cellular landscape of tumorigenesis, specific enzymes, namely matrix metalloproteinases (MMPs), emerge as pivotal contributors. They may influence this process at the level of tissue remodelling, angiogenesis, changes in cell signalling, invasion and metastasis, but may also serve as prognostic markers or therapeutic targets. Although the function of MMPs has been known for a long time, their specific role in the pathogenesis of brain tumors has only begun to be investigated in the last three decades. Deregulation of MMPs expression, frequently observed in brain tumor tissue, is associated with malignant phenotype, dependent on the grade of malignancy and associated with a worse prognosis. They participate in tissue remodelling under physiological and pathological conditions, which predetermines their action especially in the process of invasion and migration of tumor cells into the surrounding tissue. Related to their potential in tumor progression, they are also being investigated as possible targets in anticancer therapy. It would be very difficult to characterize in detail the role of all known MMPs in the context of brain tumor pathogenesis, so we have selected those that have an essential effect in this issue.

Key words: gliomagenesis, metalloproteinases, extracellular matrix

INTRODUCTION

Gliomas represent the prototypical and prevalent tumors of brain tissue, stemming from the transformation of glial cells, constituting approximately 90% of all cells within the nervous system. In 2021, the fifth edition of the WHO classification for central nervous system (CNS) tumors was published, which is the sixth updated version of the international standard for the classification of brain and spinal cord tumors. In addition to histological and immunohistochemical characterization, the latest classification is based on the irreplaceable role of molecular diagnostics in these tumors (1).

Glioblastoma multiforme (GBM) belongs to WHO grade IV glioma group and represents 53.8% of all gliomas (2). Glioblastoma is one of the most common and aggressive brain

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neoplasms with a relatively unfavourable prognosis and a median survival of only 12–15 months after the diagnosis. Surgical resection followed by radiation therapy and chemotherapy has been a conventional therapy for almost three decades. Due to the rapid growth with a high degree of infiltration into the surrounding tissues, a perfect surgical resection of GBM is still a major issue and the disease is in many cases recurrent (3). Although Temozolomid (TMZ) has been a standard chemotherapeutic in the treatment of glioblastoma, since its approval in 2005, the response to its use among brain tumor cells varies (4). The development of TMZ resistance in a significant number of patients is one of the main reasons for the failure of GBM treatment. The origin and progression of glioblastoma is multifactorial, so it involves a combination of genetic and environmental factors. In this process, specific enzymes called matrix metalloproteinases (MMPs) undoubtedly play a multifaceted role, contributing to tumor invasion, angiogenesis, and disturbances in cell signalling or immune modulation.

MMPS IN CENTRAL NERVOUS SYSTEM

A total of 23 human matrix metalloproteinases (MMPs), known so far as zinc-dependent endopeptidases, are key in the degradation of the extracellular matrix (ECM) (table 1) (5). Of these, more than 10 different types have been detected in the mammalian central nervous system at both transcriptomic and proteomic levels (6). Studies have demonstrated that MMPs exhibit a varied expression profile depending on the developmental program of neuronal tissue. Several MMPs, including MMP-2, -9, -11, -12, -13, -14, -15, and -24, show a developmental regulation, while others such as MMP-3, -7, and -10 remain unchanged during this process (7). Based on their sub-cellular distribution and specificity for components of the ECM, the MMPs are divided into membrane-type matrix metalloproteinases (MT-MMPs), collagenases, gelatinases, stromelysins, and matrilysins. The ECM, which comprises various proteins like fibronectin, thrombospondin-1, laminin, and osteopontin, holds significance in cancer progression. These ECM proteins impact the tumor behaviour by affecting cell movement and the formation of new blood vessels (angiogenesis). The interplay between cancer cells and ECM elements is crucial for various stages of cancer development, including cell transformation and carcinogenesis not only in CNS tissue (8,9).

MMPS AND BRAIN CARCINOGENESIS

Despite cancer cells produce MMPs in small quantities, they leverage their influence by stimulating neighbouring host cells to produce MMPs through the secretion of interleukins, interferons, growth factors, and other extracellular MMP inducers in a paracrine manner (10). Normal cells surrounding the cancer cells can also secrete MMPs that attach to the surface of cancer cells and can be utilized by them (11).

One of the most important metalloproteinases in the context of brain tumors are gelatinases MMP-2 and MMP-9. Gelatinases are primarily responsible for the degradation of gelatin and collagens facilitating tumor cell invasion into the surrounding healthy brain tissue. They are often overexpressed in high-grade gliomas, correlating with the increased invasive potential of these malignancies (12). Among other processes, they are also involved in the neovascularization of the tumor mass and can participate in the disruption of the blood-brain barrier (13). Dobra et al. examined the MMP-9 content of small extracellular vesicles (sEVs) from patients with tumors with a different invasion capacity. They found a relation between low MMP-9 level in sEVs and improved survival of glioblastoma patients, and MMP-9 levels showed a positive correlation with aggressiveness. These findings suggest that vesicular MMP-9 level might be a promising prognostic marker for brain tumors (14). Experiments with glioma cells U251 and nude mice showed that the expression of MMP-2

and MMP-9 in recurrent gliomas was significantly higher than those in primary gliomas and radiotherapy increased the expression of MMP-9 proving a poor prognosis in glioma recurrence. This suggests that MMP-9 may be an important target also in radiosensitization of gliomas (15). MMP-2 and MMP-9 have been also shown to present an increased activity in cortex neuronal nuclei after focal cerebral ischemia. Their increased gelanolytic activity in nucleus occurs to be linked with MMP-dependent cell death triggering neuroinflammatory reactions (16).

Collagenase MMP-13 plays a crucial role in initiating the invasive progression of glioma due to its proteolytic activity. Its expression is notably higher in glioma compared to the surrounding normal brain tissue, especially in advanced grades of glioma. Some researchers propose MMP-13 as a potential biomarker for tracking the progression of glioblastoma (GBM). A stimulation of a highly invasive glioma cell line U251 in vivo with endothelin resulted in an increased expression of MMP-13, MMP-9, and enhanced cell migration. The addition of MMP-13 and MMP-9 inhibitors successfully mitigated this heightened cell migration (17,18).

ECM-degrading enzymes play a significant role in influencing the survival of metastatic cells by modulating the process of apoptosis. In particular, MMP-7 contributes to the survival of tumor cells by cleaving the Fas ligand. This cleavage action removes the ligand from the cell surface, thereby preventing it from stimulating the Fas death receptor. The Fas death receptor is a powerful mediator of innate apoptotic pathways (19). By evading apoptosis through this mechanism, malignant cells not only escape cell death but also potentially develop a resistance to chemotherapeutic treatments.

Matrix metalloproteinase-14 (MMP-14), also recognized as membrane-type matrix metalloproteinase 1 (MT1-MMP), is attached to the cell membrane and possesses the ability to activate other MMPs. It specifically activates proMMP-2 directly and indirectly influences MMP-2 and MMP-9. MMP-14 can enzymatically break down potent inhibitors of central nervous myelin, including BN-220. Moreover, MMP-14 is capable of digesting proteins with adhesion functions. Interestingly, MMP-14 is not limited to extracellular processes; it is also involved in intracellular activities. It passes along the tubulin cytoskeleton and plays a role in intracellular recycling pathways. Abnormalities in MMP-14 expression are associated with mitotic spindle aberrations and chromosomal instability, ultimately leading to a malignant transformation of neoplastic cells (20).

An important key element of tumor cell growth and proliferation is angiogenesis. This process can be stimulated by various signalling molecules produced by tumor cells or by surrounding tissue. MMPs play a significant role in the formation of new blood vessels to supply nutrients and oxygen to the growing tumor. Malignant cells and endothelial cells release MMPs to remodel the ECM, creating a path for new blood vessels to grow. They contribute to the release and activation of various angiogenic factors, such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF), both potent inducers of angiogenesis often bound to the ECM (21). One of the extensively studied MMPs involved in angiogenesis is the aforementioned MMP-14. MMP-14 is a key effector in the generation of pro-angiogenic factor VEGF. It interacts with cell surface molecules such as CD44 and sphingosine 1-phosphate receptor 1 (S1P1), promoting endothelial cell migration. Furthermore, MMP-14 is crucial in the proteolytic degradation of anti-angiogenic factors like decorin. Moreover, there is an evidence suggesting that MMP-14 can degrade pro-transforming growth factor-beta (pro-TGF-β) and endoglin (TGF-β receptor), indicating its pivotal role in vessel maturation and angiogenesis, respectively (22). Additionally, MMP-14 seems to be indispensable in determining ECM adhesion and the formation of tubes by human endothelial cells through the modulation of MMP-2 expression. This underscores its significant involvement in regulating angiogenesis-related functions in human endothelial cells (23).

The increased expression of various MMPs observed in brain tumors is the result of deregulation of several intracellular signalling pathways that have long been a subject of increased

interest. MMP-2 has been found to have intracellular activity and play a role in processes occurring in the cell nucleus. MMP-2 directly interacts with p21 activated kinase 4 (PAK4) which aberrant expression was found to be associated with an enhanced tumor progression in various carcinomas. Complex PAK4/MMP-2 is supposed to regulate integrin mediated pathways in gliomas and earlier study revealed that MMP-2 knock down glioma cells entered on apoptosis pathway (24,25).

MMPS AS TARGETS FOR ANTICANCER THERAPY

Many MMPs have become interesting candidates for diagnostic tools and therapeutic interventions in the context of cancer due to their key involvement at virtually every critical stage of tumor development. MMPs modulation can be approached through three primary strategies: transcriptional regulation, activation control, or direct inhibition. At the transcriptional level, the interference with extracellular factors, such as interferon, and the blockade of signal transduction pathways like MAPK or ERK can effectively hinder MMPs synthesis. Critical to MMPs inhibition is the modulation of nuclear transcription factors, including NF-κB or AP-1 (26). Another key aspect of MMPs regulation is their activation process, given that they are initially secreted as inactive zymogens. Monoclonal antibodies targeting MMPs are considered an effective means of inhibiting their activation. Such antibodies have been successfully developed, for example, against gelatinase B (MMP-9) as well as for MT1-MMP (MMP-14) (27,28). In healthy organisms, MMP activity is regulated by endogenous TIMPs that are natural inhibitors of MMPs. High TIMPs levels lead to ECM accumulation due to inhibition of the degradation processes, whereas low TIMPs activity results in elevated proteolysis (29). TIMPs can also inhibit the growth, invasion and metastasis of malignant tumors. On the other hand, there are non-specific inhibitors such as α1-proteinase inhibitor and α2-macroglobulin that can affect the regulation of MMPs activity (30).

Another strategy to exploit the potential of MMPs is their ability to recognize and cleave peptides with specific sequences, making it a hot spot for targeted drug release studies (31). In a recent study dealing with the treatment of GBM, MMP-2 was used for this purpose, precisely because of its high expression in glioma tissue (32).

Despite encouraging results from preclinical studies targeting MMP inhibition as a potential cancer treatment, the results of phase III trials were ultimately disappointing. The lack of success can be attributed to the inadequate design of the clinical trials, the specific properties of the MMP inhibitors used, the limited understanding of the complex nature of MMPs, and the differences between the results observed in mouse models and the patient populations participating in the clinical trials (33). It is therefore extremely important to show interest in extending the current knowledge of these multifactorial enzymes.

CONCLUSION

In recent years, matrix metalloproteinases (MMPs) have revealed novel biochemical properties that exhibit both extracellular and intracellular activities, including intranuclear functions. These activities have been implicated in the invasiveness of brain tumors, particularly gliomas. Understanding the integral connection between MMP function and essential cellular processes, such as apoptosis, cell migration, and angiogenesis – frequently implicated in glioma pathogenesis – presents MMPs as potential tumor markers critical for developing targeted therapies.

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Table 1 Classification of matrix metalloproteinases (MMPs)

Subgroup	MMP number	Nomenclature
collagenases	MMP-1 MMP-8 MMP-13	Collagenase-1 Collagenase-2 Collagenase-3
gelatinases	MMP-2 MMP-9	Gelatinase-A Gelatinase-B
stromelysins	MMP-3 MMP-10 MMP-11 MMP-12	Stromelysin-1 Stromelysin-2 Stromelysin-3 Metalloelastase
matrilysins	MMP-7 MMP-26	Matrilysin-1 Matrilysin-2, Endometase
membrane type MMP	MMP-14 MMP-15 MMP-16 MMP-17 MMP-24 MMP-25 MMP-23	MT1-MMP MT2-MMP MT3-MMP MT4-MMP MT5-MMP MT6-MMP CA-MMP
others	MMP-19 MMP-20 MMP-18 MMP-22 MMP-27 MMP-28	RASI-I Enamelysin Epilysin

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CHARACTERIZATION OF PLASMIDS ISOLATED FROM BACTERIAL FLORA OF CRC PATIENTS – PROOF OF CONCEPT

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Abstract

Introduction: Colorectal cancer (CRC) is one of the leading oncogenic disorders, both in terms of incidence and mortality. The etiology of the disease is certainly multifactorial. Various risk factors like alcohol consumption, smoking, CRC family history, inflammatory bowel disease, hormone therapy, aspirin/nonsteroidal anti-inflammatory drugs use, higher body mass index, consumption of red and/or processed meat, insufficient physical activity, and decreased intake of fruit and vegetables have been pointed out; however, there is not enough support evidence for a single particular causative mechanism. Recently, gut bacterial microbiota has been shown to influence significantly the pathogenesis of CRC. However, little attention is paid to the putative impact of plasmids in gut flora. **Material and methods:** We have designed and tested the workflow for semi-selective isolation and amplification of random circular sequences. The exploitation of rolling circle amplification (RCA) with a random hexamers protocol is crucial for the outcome. **Results:** Our results suggest that it is possible to isolate and amplify plasmid DNA from gut flora and further process, sequence, and identify them. **Discussion:** Little is known about the interactions between bacterial plasmids and human cells. The collection of plasmid sequencing data and the comparison of CRC patients and healthy control sequences can be the first step to elucidating this phenomenon.

Keywords: CRC, gut bacterial flora, plasmids

INTRODUCTION

Colorectal cancer (CRC), with between one and two million new cases every year, is undoubtedly one of the most common oncological diseases worldwide (1, 2). It is the third most common cancer diagnosed and the fourth most common reason for cancer-associated death (1,3). The global distribution of newly diagnosed cases is unequal, and the incidence rates are highest in developed countries; however, due to the "westernization" of diet and lifestyle in developing countries, it is expected to significantly increase the global burden of disease by 2030 (4). Also, an alarming growing trend in early-onset CRC in young individuals has been observed in recent years. Particularly, the incidence in individuals under 50 has been increasing by 2% per year (5). Nevertheless, this observation can be partially attributed to the rapid development of new approaches to early screening (6).

The pathogenesis of colorectal cancer is multifactorial. Several mutations in genes, such as *APC, KRAS, BRAF, P53, PIK3CA, SMAD4, ARID1A, MYC,* and others, have been associated with the development of CRC (7–11). Also, the involvement of lncRNAs like GLCC1, LINRIS, and many more have been described (12–14).

Both lifestyle and genetic factors play an important role in the etiology of CRC. The influence of nutrition on initiating the pathological process has been studied extensively

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(3,15). Presumed risk factors associated with the development of CRC include alcohol consumption, cigarette smoking, CRC family history, inflammatory bowel disease (IBD), former and current postmenopausal hormone therapy (HT), aspirin/nonsteroidal anti-inflammatory drugs (NSAIDs) usage, higher body mass index (BMI), frequent consumption of red and/or processed meat, insufficient physical activity (PA), and a decreased intake of fruit and vegetables (16, 17). However, the precise causative factors are yet unknown. For example, eating white meat or fish is not associated with a high risk and may even reduce the incidence of CRC. The influence of saturated fat, protein, iron, heterocyclic amines produced by cooking, N-nitroso compounds, and increased levels of bile acids in the colonic lumen has been considered (18). The work of zur Hausen outlined a hypothesis that not directly the consumption of undercooked red meat but the presence and survival of certain – for now elusive - causative agent in undercooked meat may play an important role in pathogenesis (19).

Lately, another factor - gut bacterial flora - has gained more attention. Indeed, it has been shown that the species composition and the metabolic activity of the intestine flora of CRC patients differ significantly from those of healthy controls (20–22). The correlation between the gut microbiome status and the presence of CRC was shown to be sensitive and specific enough to serve as a reliable biomarker for this malignancy (23). On the other hand, gut microbiota can have a protective effect. For example, gut flora can enhance the immune system response via the stimulation of chemokine production by CRC cells, thus increasing the recruitment of beneficial T cells into tumor tissues (24). Also, the species *Clostridium butyricum* was shown to reprogram the proliferation, migration, stemness, and tumor growth in CRC by regulating pivotal signal molecules, including MYC (25).

The recent discovery of viroid-like elements denoted “obelisks” illustrates that not enough attention is paid to the structures that are hosted by bacteria. These entities remained unnoticed despite being widely spread in human colonizing microbiota (26). Needless to say, plasmids are generally known to be present in bacteria, archaea, and even some eucaryotic organisms, and they are classified into several classes by their function; however, not much attention is given to this fact in the context of a possible interaction with cells of the human intestine tract. Few studies analyzed meta-genomic data and suggested particular plasmids to be used as biomarkers (27).

Little is known about the possible interactions between bacterial plasmids and human cells. Nevertheless, a couple of observations might support our aim to investigate this phenomenon further: I) As mentioned above, it is generally accepted that bacterial metabolomes in the gut can influence the pathogenesis of CRC. Also, it is known that plasmids can provide a whole spectrum of additional properties to bacteria. II) Gut is a specific space where the massive intake of various molecules is localized and optimized. III) Some human tumorigenic viruses like HPV, KSHV, or EBV replicate themselves in a plasmid-like manner in human cells (28); thus, the replication of bacterial plasmids even in human cells is not excluded. IV) Tumor-inducing plasmids (Ti-plasmids) are relatively common in the plant realm (29, 30).

The aim of this preliminary study is to provide a proof of concept that random plasmids from gut bacteria can be isolated and amplified by means of the RCA method. Our previous work has shown that such amplified products (obtained from other sources) can be further processed, digested, cleavaged, cloned, and sequenced (31). However, the collection of plasmid sequencing data and their comparison between CRC patients and healthy controls would be just a first step to elucidate this possibly existing interaction network.

MATERIAL AND METHODS

The design of workflow for semi-selective isolation and amplification of random circular sequences from intestinal flora is illustrated in Figure 1.

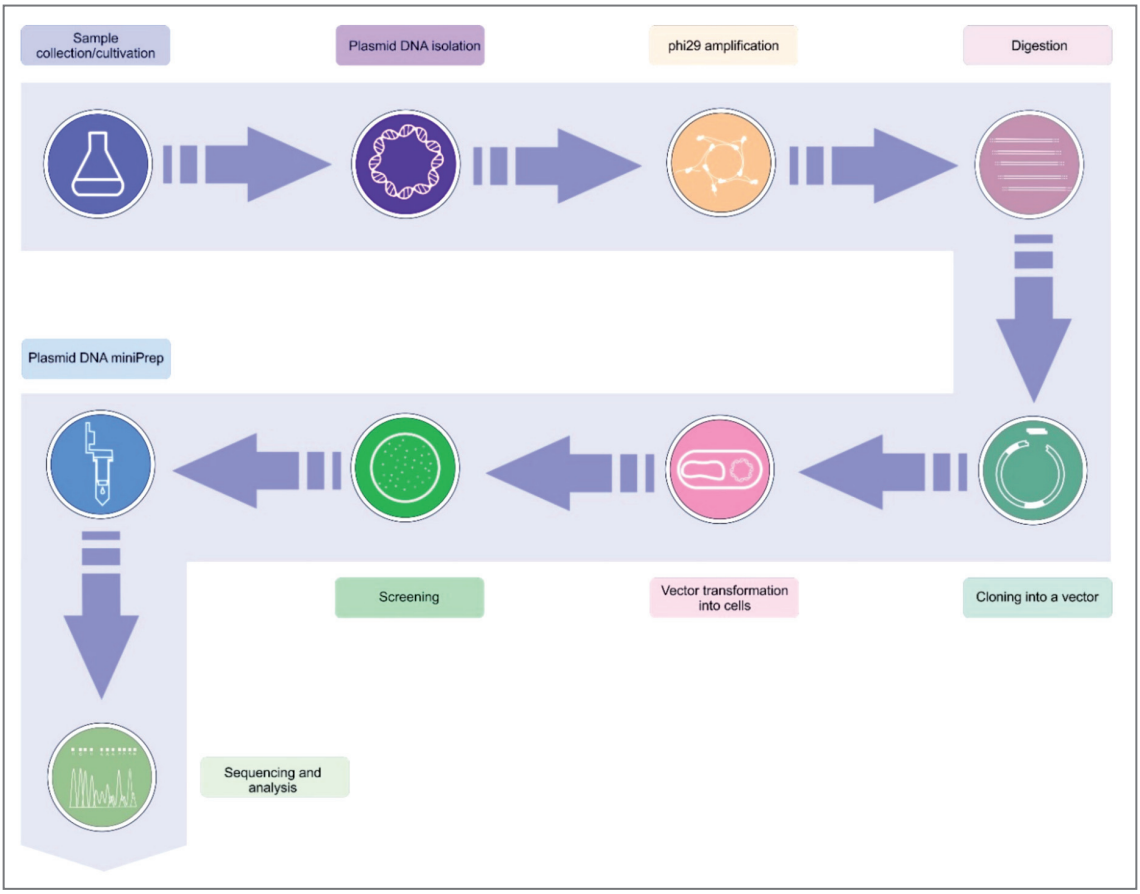

Fig. 1 Proposed workflow for the isolation of plasmid sequences from column bacterial flora.

Sample cultivation:
Stool samples were cultivated in LB (Invitrogen) without antibiotics at 37 °C overnight, shaking 200 RPM. Plasmid DNA isolation was performed with PureLink™ Quick Plasmid Miniprep Kit (Invitrogen) according to the manufacturer´s protocol.

Isolation of circular DNA from gut microflora:
The mixture of the isolated plasmids DNA served as a template for *Phi29* DNA polymerase (New England Biolabs). Exo-resistant random hexamers (Thermo Scientific™) were used as primers. First, the mixture of *Phi29* polymerase buffer, hexamers, and DNA in water was incubated at 95 °C for 3 minutes, then cooled down to 4°C to allow the separation of DNA double-strand and primer annealing. After primer annealing, the mixture of thermolabile *Phi29* polymerase, *Phi29* polymerase buffer, another half of hexamers, BSA, and dNTPs diluted in water was added and incubated at 30 °C for 18 hours; then the product was heat inactivated at 65 °C for 10 minutes and cooled down to 4 °C.

Cleavage of RCA product:
Subsequent cleavage of the products was performed using restriction endonucleases (New England Biolabs) with belonging buffers at 37 °C for 1 hour. The following enzymes from pUC19 MCS were selected and used: *BamHI*, *EcoRI*, *HindIII*, *KpnI*, and *XbaI*.

Electrophoretic separation of digested DNA fragments:
Electrophoresis allows the separation of digested DNA fragments according to size. The 1.5% gel was prepared from Certified PCR Low-melt Agarose (Bio-Rad) and TAE solution (Thermo Scientific). Midori Green Advance (NIPPON Genetics) was used for DNA staining.

Isolation of DNA fragments from the gel:
Cleaved DNA fragments were cut out from the gel and isolated using NucleoSpin Gel and PCR Clean up kit (Macherey-Nagel) according to the manufacturer´s protocol.

Cloning of the isolated fragments into the vector pUC19:
Isolated cleaved-out fragments were cloned into the pUC19, opened with the respective restriction enzyme, and dephosphorylated with FastAP (Thermo Scientific) using Rapid Ligation Kit (Thermo Scientific).

Transformation of ligation products into competent bacteria:
Further, ligation mixtures were transformed into DH5α competent bacteria (Invitrogen) plating on agar plates supplemented with ampicillin (AMP) (Gibco). Colonies were grown overnight, at 37 °C, in the bacterial incubator.

PCR screening:
Bacterial colonies were screened for positive clones using Go Taq G2 Green Master Mix (Promega), with in-house designed primers pUC19-seq-F and R (5´ TGGAATTGTGAGCG-GATAAC 3´ and 5´ ATTAAGTTGGGTAACGCCAG 3´) (Integrated DNA Technologies). After electrophoretic separation, clones producing fragments of the expected size were selected.

Miniprep isolation:
Selected colonies were grown in LB supplemented with AMP, at 37 °C overnight, shaking 200 RPM. Again, plasmid DNA isolations were performed with PureLink™ Quick Plasmid Miniprep Kit, during the procedure identical to sample processing.

Sequencing:
Isolated DNA sequences were sequenced using an in-house sequencing assay and the same primers that were used for the PCR screening.

Data analysis:
For the archiving, identification, comparison, and analysis, free software, namely: Blast (National Library of Medicine), SerialCloner 2-6-1, and Chromas 1.45, were used.

RESULTS

In order to test the suitability of the designed workflow, we performed sample collection, cultivation, isolation of small circular bacterial DNAs, RCA isothermal amplification with random primers, and electrophoretic analysis. Fig. 2 shows the outcome of the procedure. The line 1. with negative control shows no sign of massive DNA production. On the contrary, line 2. demonstrates massive amplification in the RCA reaction with the template DNA isolated from bacteria. Most DNA products are of the size of several 10 kbp; a smaller fraction of amplified DNA wasn´t able to lease the well due to its size. Positive control represented by plasmid pUC19 contained amplified DNA, of which the majority gave band between 1500 and 5000 bps after treatment with EcoRI, corresponding with pUC19 size of 2686 bp.

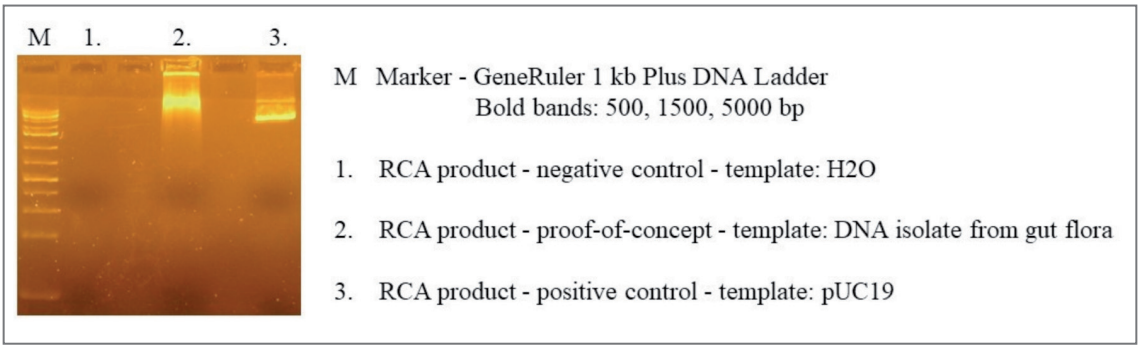

Fig. 2 Results of RCA amplification with the presumably circular DNA template isolated from gut bacteria visualized on the electrophoretic gel

We have also optimized and trouble-shot further downstream steps of the protocol. However, this part was already tested and published previously with the template obtained from bovine serum (31). (Data not shown.)

DISCUSSION

The aim of this preliminary study was to test the protocol modified for the isolation of DNA sequences, preferably from plasmids of gut bacteria. Several issues deserve consideration. For example, the cultivation of a sample can influence the ratio of particular bacterial species. The ones that are hard to promote in LB media would be underrepresented. Thus, direct isolation from a sufficient amount of sample shall be considered.

The concept of this project is based on these specific facts: I) *Phi29* polymerase is known for an effective amplification of circular molecules; therefore, plasmids, circular viral genomes, and other circular molecules are multiplied preferably (32). II) Strand displacement activity, high fidelity, and extreme processivity of *Phi29* polymerase allow a highly accurate generation of large fragments during the isothermal reaction. Thus, the product is composed of multiple copies of circular template sequences linked in linear DNA fragment large up to 100 kbp (33). III) The random sequence of six nucleotides can be expected to repeat every 4096 nucleotides (4^6) in unknown DNA. Most of the restriction endonucleases recognize six nucleotides long palindrome. Thus, it is reasonable to speculate that at least some from the set of enzymes recognizing six nucleotide sequences will cleave unknown DNA, resulting in the fragment and/or fragments long several hundreds or thousands of bps. Naturally, the increased number of enzymes used increases the chance of capturing and identifying more sequences, thus expanding the database of sequences.

For the sake of simplicity, we used the pUC19 vector with selected restriction enzymes; however, any suitable vector with an appropriate set of enzymes could be used.

Provided that the isolation of whole circular sequence is needed or desired, long PCR with back-to-back primers can be designed based on the partial sequence.

Using this approach, valuable data with the potential to describe the differences between the plasmids of bacteria in healthy controls and CRC patients or even elucidate the eventual role of plasmids in the etiology of CRC can be gained. Further investigation is needed. This proof of concept shall serve as a basis for the grant application.

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OPTIMIZING DROPLET DIGITAL PCR ASSAY FOR PRECISE ASSESSMENT OF MEIS1 GENE PROMOTER METHYLATION

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Abstract

DNA methylation is characterized as a gene regulatory mechanism that involves the methylation of the 5-carbon (C5) position of cytosine, resulting in the formation of 5-methylcytosine. The analysis of aberrantly methylated cytosine-phosphate-guanine (CpG) dinucleotides, primarily in the promoter regions of tumor suppressor genes, can serve as promising prognostic and predictive markers of cancer development. Meis homeobox 1 (*MEIS1)* gene, crucial for cell growth and differentiation, exhibits dysregulation linked to various cancer types, acting as both a positive and negative regulator. The selection of an appropriate method for the evaluation of gene promoter methylation status is important for clinical implementation without biases regarding false positive and false negative outcomes. The study focuses on the optimization of a novel droplet digital PCR (ddPCR) assay for identifying the methylation status of *MEIS1*. Compared to traditional methods, ddPCR offers an increased sensitivity and specificity, presenting a promising tool for precise DNA methylation assessment with potential implications for cancer diagnostics and prognostics.

Keywords: methylation, *MEIS1*, ddPCR, primer design

INTRODUCTION

Epigenetic modifications, including DNA methylation, are heritable changes essential for normal development via the control of gene expression (1). These heritable changes are established during the cell cycle and cell division, leading to distinct identities of gene expression profiles while the primary sequences of DNA are the same. Failure in the proper maintenance of epigenetic machinery can lead to an alteration of gene expression and subsequent carcinogenesis (2). Recent evidence in the field of epigenetic analysis showed that cancer cells exhibit global epigenetic alterations in addition to various genetic changes observed during malignant transformation (3). DNA methylation is one of the most extensively studied epigenetic modifications characterized as the methylation of C5 position of cytosine, resulting in its modification into 5-methylcytosine. DNA methylation occurs mainly

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at the cytosine-phosphate-guanine (CpG) sites, which are localized in the promoter region of tumor suppressor genes. The transcription of genes initiates from the promoter regions characterized by relatively high frequencies of CpG dinucleotides. For this reason, aberrantly methylated CpG islands are currently defined as promising prognostic and predictive markers associated with cancer development (4).

Meis homeobox 1 (*MEIS1*) regulates cell growth and differentiation during vertebrate development. Many studies observed that *MEIS1* dysregulation is correlated with the development of numerous cancer types. Two documents emphasized the role of *MEIS1* in cancer development. *MEIS1* can act as a positive regulator of cancer proliferation observed in leukemia (5,6) or a negative regulator of several other solid tumors (7,8). The mentioned dysregulation of *MEIS1* expression suggests to be a consequence of the increased promoter methylation as was described in colorectal cancer (9).

Selecting the appropriate methods for the evaluation of gene promoter methylation status based on their capacity to distinguish 5-methylcytosine and nonmethylated CpG sites is the most crucial step toward subsequent implementation of the detecting method into clinical practice, particularly without biases regarding false positive and false negative results. Currently, several innovative methods and high-throughput molecular biology and genomics technologies are available to detect the degree of DNA methylation status (10). PCR-based methods have been widely used to evaluate DNA methylation status for decades. The first generation of PCR used methylation-specific-restricted enzymes to distinguish methylated and unmethylated alleles. These methods were followed by methylation-specific PCR (MSP) based on the amplification of sodium bisulfite-treated DNA and consecutively analyzing the presence or absence of converted unmethylated cytosine to uracil after the process of deamination (11). Droplet digital PCR (ddPCR) is a relatively novel method for estimating the absolute quantification of the target sequence of DNA. Recent data show that ddPCR manifests higher sensitivity and specificity than routinely used qPCR (12). In the present article, we describe the optimization of in-house developed ddPCR assay for the identification of the *MEIS1* methylation status.

MATERIAL AND METHODS

Designing methylation PCR primers and probes

Designing of ddPCR was conducted by the employment of free on-line application: The Eukaryotic Promoter Database (EPD), MethPrimer, and Primer3.

Firstly, EPD application was used (https://epd.expasy.org/epd) to find promoter regions of *MEIS1*. The nucleotide sequence with transcription start sites (TSS) (chr2: 66,434,560 - 66,435,484) is presented in Figure 1.

GGTCTGCAGAAAGCCCGAGCCACCTCAACCCCATGTTCTCAGGACTCCTTAGCAGAGGCTTTCCCAACCTGGCTTCTCCCTCCTTTTCCTC
CACGATCCCGCTTTGACTTTTCTCCTTGCAGTGTTTAGTTCTGAGAATTCAGTACTTTGCGCATCACCCCCTGCCCCGAAAAACACTGGCA
GACCCCAATAATTTCGAGGAAAGTCATGAAGTCTATGCGCGGAGCCCTGTGCAAAATAACTCCCGCTGCTGCCTGCCCGGCGTTGATTCCC
AATTTATTTCAAGAGAGTCGGCTTTGGGGAGAGAGTCTGCAGGGGGAGGGAGAGAAAAGAATACTGAAAATAAAGCTGGCGGCCGCG
GGCTACTGCCTGCGTTTGTGTGCGTGTGCCCTGGGTGTGTGGTGTTTGTGCCTGGGCGTGCGATTTAATGGAGCGCCTCTCTGCCTCTCC
AGTGCGGCCAGAGCTCGCTTCGCGCACCCACCCCTGCCGAGGAGCCTACTTGCTGCAGCCCAATGCATTGTGTAAGACGCGACCTGTTAT
GGCCACCACTACTTCCGGGTTCTAGCATTCTGGTCGGAATCCACCTCTCCGCCTGTGCAACACACACTTTACACACGCACGGGGACTGCA
AGCGGGCAGCATCGATCGTGGCTCCTTTAAGACAAACTCAGACAGACATTTTTTTTAACCCTCCTTCTCTAATCTCCTTCCAGTGCAGCACT
TGCAAAGAGGGAGAGAGAGGGAGAGAAAGAAAGAGAGAGAGAAGAGAGAAACTGATTAGGAATTAGGACTGATTCAAGGGAAG
CGAGCGCTAGGGCTTTGTGCATTTGAATATTAACATTTGAGGTGTTCTGACCAGAAGAAGACAGAGCGGATGATCATTCATTCACCACGTT
GACAACCTCGCCTGTGATTGACAGCTGG

Fig. 1 Promoter region of *MEIS1* with marked TSS

Consequently, we used the commercially available software MethPrimer (https://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) to perform digital bisulfite conversion of the input sequence with predicted CpG islands. Bisulfite converted promoter region with CpG islands (upstream to TSS) are shown in Figure 2.

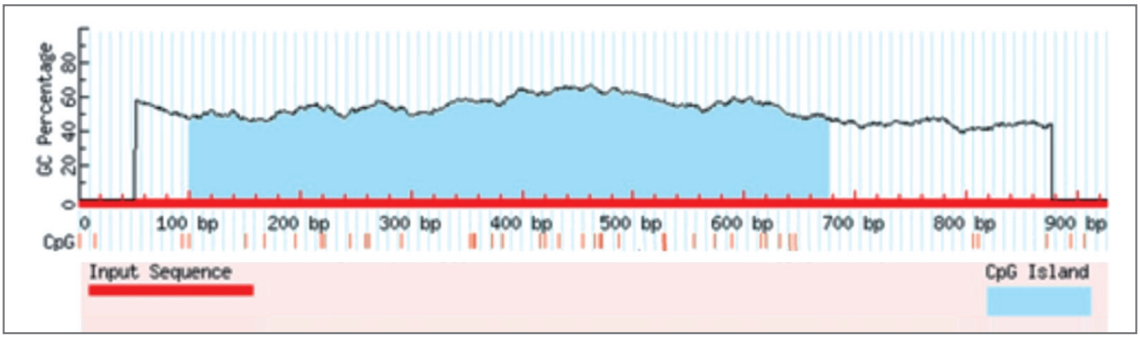

Fig. 2 CpG island prediction generated by MethPrimer software

The final step included usage of free available primer design software Primer3 (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) in order to select specific methylation-independent primers (MIP) with specific methylated (labeled by FAM-BHQ1) and unmethylated (labeled by HEX-IBFQ) probes. Our designed probes targeted four nearby CpG sites (chr2:66,434,908; chr2:66,434,912; chr2:66,434,914; chr2:66,434,928) in the *MEIS1* promotor region. The primers' and probes' sequences are presented in Table 1.

Table 1 Primer and probe sequences for *MEIS1* detection

Primers	
Name	Sequence (5´→3´)
MEIS1_1 Forward	TGGGGAGAGAGTTTGTAGG
MEIS1_1 Reverse	ACACAAACACCACACACC
Probes	
Name	Sequence
MEIS1_1 Methylated probe (FAM-BHQ1)	CGGTCGCGGGTTATTGTTTGC
MEIS1_1 Unmethylated probe (HEX-IBFQ)	TGGTGGTTGTGGGTTATTGTTTGTGT

ddPCR analysis

Optimizing PCR conditions

The ddPCR platform (Bio-Rad Laboratories, Hercules, CA, USA) was used for the optimization of methylated (M-Probe, FAM labeled) and unmethylated (UnM-Probe, HEX labeled) probes. As a template sequence, we used commercially available fully methylated and fully unmethylated EpiTect DNA controls (Qiagen, Hilden, Germany). The reaction mix for ddPCR

contained 10 µL of Supermix for Probes (No dUTP) (Bio-Rad Laboratories), 0.45 µL of each primer, 0.45 µL of each probe, 1 µL of methylated and unmethylated control DNA adjusted with 7.75 µL of water up to a final volume of 20 µL. Subsequently, 70 µL of Droplet Generation Oil for Probes, along with the reaction mixture, was loaded into a DG8 cartridge and inserted into the QX200 Droplet Generator (Bio-Rad Laboratories) to divide each sample into 20,000 droplets. After generating oil emulsion, approximately 40 µL of the sample was transferred into 96-well PCR plates, covered with a pierceable foil, and heat-sealed by Bio-Rad's PX1 system. The first optimization step of the ddPCR assay covers estimating optimal PCR conditions. The thermal PCR consisted of enzyme activation for 10 min at 95 °C, followed by 40 cycles of denaturation for 30 sec at 94 °C, annealing/extension step for 1 min with temperature gradient, finished by one cycle of enzyme deactivation for 10 min at 98 °C. To define the optimal conditions of annealing, we performed the above-mentioned temperature gradient in the range of 50 – 62 °C. After finishing the PCR program, we loaded the plate onto the QX200 Droplet Reader (Bio-Rad Laboratories) for a final analysis.

Concentration gradient

The second optimization step was performing a concentration gradient of eight different dilutions of DNA controls to confirm the dynamic range and linearity of the method. The reaction mix for ddPCR contained 10 µL of Supermix for Probes (No dUTP) (Bio-Rad Laboratories), 0.45 µL of each primer, 0.45 µL of each probe, diluted methylated and unmethylated DNA controls in water with 8000, 4000, 2000, 1000, 500, 250, 125, and 62 copies per reaction adjusted with a variable volume (regarding to DNA input) of water up to a final volume of 20 µL. Further workflow and analysis were the same as described above.

RESULTS

Estimating optimal PCR conditions

Using commercial methylated and unmethylated DNA, we determined the best annealing temperature to be 57.1 °C for both methylated and unmethylated probes. Both DNA controls were combined with a methylated probe and an unmethylated probe. The methylated probe (Fig. 3A) manifested a sufficient sensitivity in the detection of methylated DNA and, at the same time, a perfect selectivity capacity. The unmethylated probe showed a high sensitivity for detecting unmethylated control DNA, but the same probes also manifested a cross-reactivity resulting in the generation of positive droplets detected in methylated control DNA (approx. 9%) (Fig. 3B).

Concentration gradient

Eight different dilutions of DNA controls were included in the concentration gradient to confirm the dynamic range and linearity of the method. A methylated (Fig. 4A) and an unmethylated DNA control (Fig. 4B) were used for the dilution. Similar to the concentration gradient, both controls were combined with a methylated probe and an unmethylated probe. The optimal concentration was determined by considering signal amplitude, the overall count of positive droplets, and binding specificity. In addition, thresholds were set at the level of 1500 for FAM and 1400 for HEX.

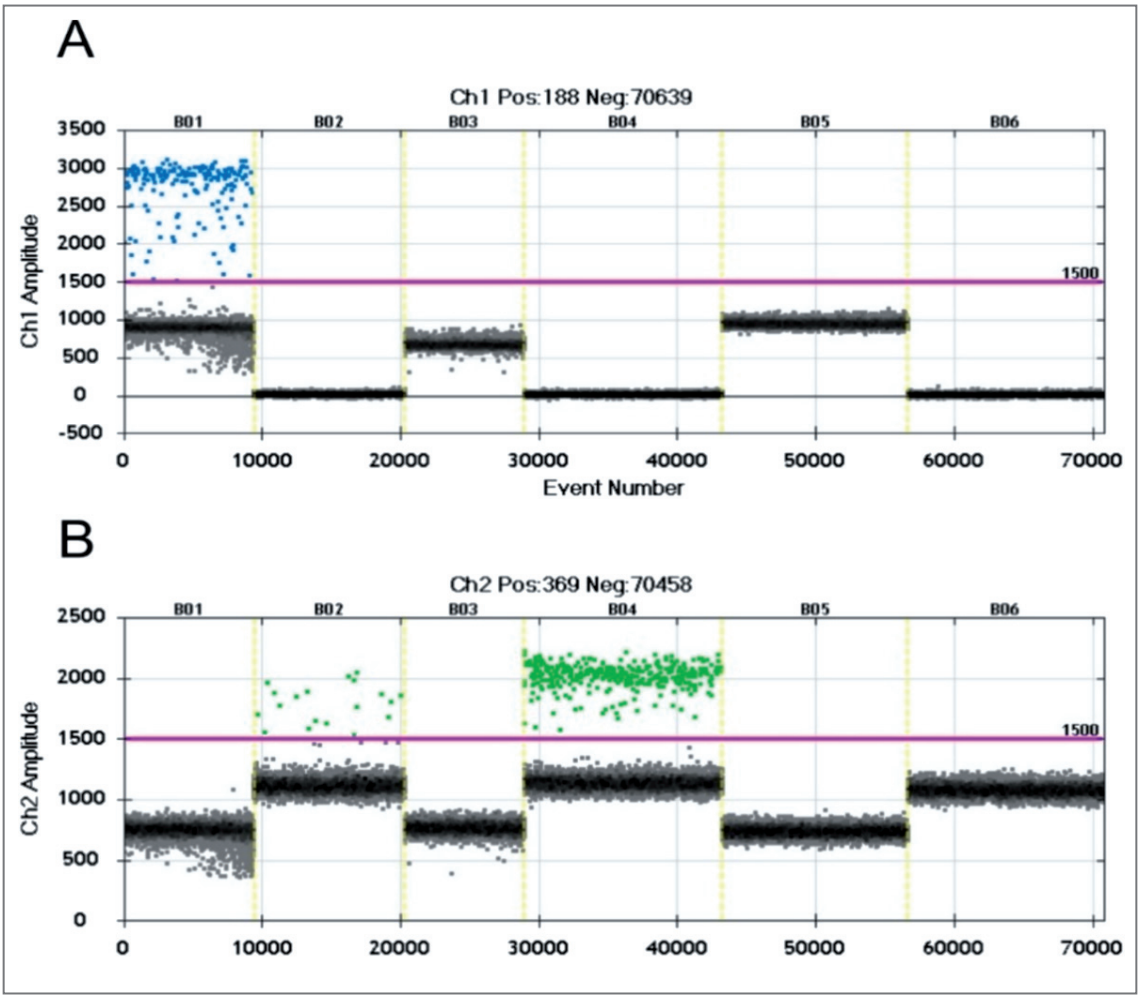

Fig. 3 PCR optimization. Figure 3A shows a positive signal generated by methylated probes using methylated control DNA (column B01); Figure 3B shows a positive signal generated by unmethylated probes using unmethylated control DNA (column B04). The positive signal generated by unmethylated probes in line B02 demonstrates the cross-reactivity of unmethylated probes with methylated control DNA.

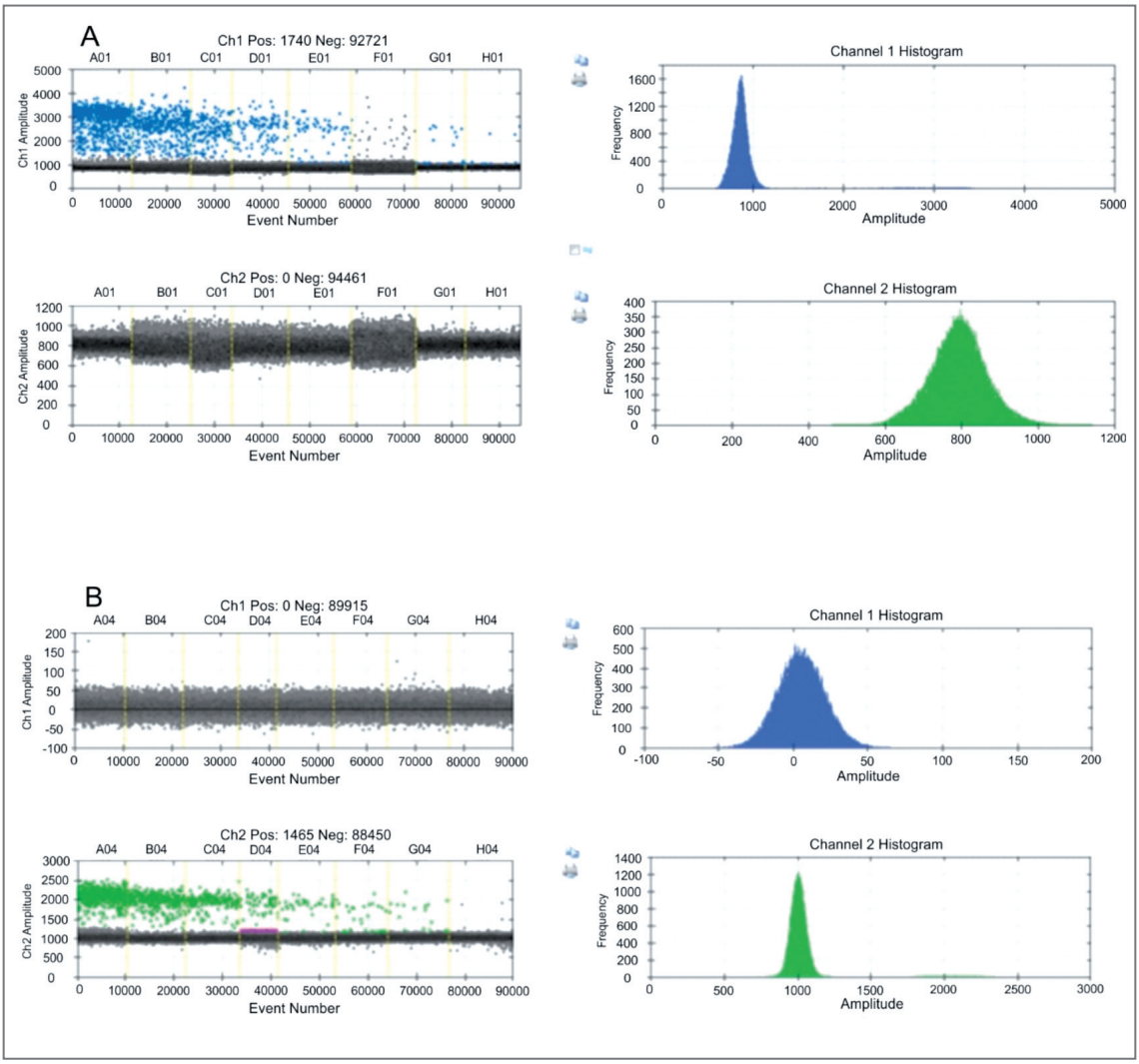

Fig. 4 Concentration gradient: A) methylated DNA control using the methylated probe. B) unmethylated DNA control using the unmethylated probe.

DISCUSSION

DNA methylation plays an essential role in cancer initiation, progression, and development of its metastatic form (13). Development and optimization of novel methods for the analysis of specific methylation patterns provides new opportunities and suggests a potential concept for clinical implementation (10). The ddPCR platform allows the precise detection and absolute quantification of targeted DNA sequences. Usage of bisulfite-treated DNA and subsequent application of specific probes for methylated and unmethylated sequences is also suitable for quantifying methylated DNA at single-base resolution utilizing the ddPCR platform (11,14).

In current study, we designed the probes with high specificity and sensitivity targeting the promoter region of the *MEIS1* gene. *MEIS1* promoter region was found using the biological

database and web resource of gene promoters EPD (15). The critical step in the methylation analysis using PCR-based methods to obtain adequate results is based on the design of optimal primers and probes targeting the gene region of interest. Nowadays, several web applications such as BiSearch (16), MSPprimer (17), or MethPrimer (18) have been developed for this purpose. In our research, we used MethPrimer to perform digital bisulfite conversion of the input sequence with predicted CpG islands. In order to design primer sequences to amplify bisulfite-modified DNA we used free-available Primer3 software (16). The software was also used to design probes in two variants to discriminate the methylation profile. Our in-house designed methylated probes recognized four CpG sites located in the promoter region. The observed data revealed that methylated probe demonstrated a good detection capacity and, at the same time, a perfect selectivity to distinguish methylated from unmethylated DNA sequences. On the contrary, unmethylated probes showed a low cross-reactivity (approximately 9%). Although the cross-reactivity of unmethylated probes was identified, these data were irrelevant for our purpose due to the fully discriminating capacity of the probe for methylated sites.

In conclusion, we described the design and optimization of the ddPCR assay recognizing methylated CpG dinucleotides in the *MEIS1* promoter region. The high sensitivity and discriminative power of our in-house developed assay proposes a potential tool for determining methylation profile of various cancer types in experimental research and subsequent clinical application.

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MOLECULAR PATHOLOGY OF HEMATOLOGIC MALIGNANCIES

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Abstract

Hematological malignancies represent a heterogeneous group of diseases that may have overlapping clinical manifestations. Successful and optimal management depends on early and accurate diagnosis of the disease. Differential diagnosis therefore requires methods of morphology, immunohistochemistry, flow cytometry, and also molecular and cytogenetic examinations. Molecular diagnostic techniques are becoming more accurate and sophisticated, which is why nowadays hematopathological diagnosis relies heavily on molecular and cytogenetic analyses. They are beneficial not only for diagnosis, but also for evaluating prognostic and risk markers, as well as treatment monitoring. This article presents an overview of selected hematological malignancies – mature lymphoid neoplasms, multiple myelomas, myeloproliferative neoplasms, myelodysplastic syndromes, and acute myeloid leukemias; their molecular pathology, risk and prognostic markers. Understanding of the biologic basis leds to a targeted therapy development for the treatment of these diseases.

Key words: hematologic malignancies, molecular pathology, recurrent mutations, genetic alterations

INTRODUCTION

Hematologic malignancies are myeloid and lymphatic tumors caused by a disruption of normal hematopoietic function (1). This heterogenous group of diseases differs in cellular origin and clinical manifestation. Classification system combines clinical, pathologic, and molecular features of the diseases. In general, they can be divided into leukemias and lymphomas. While leukemias involve peripheral blood and are composed of immature hematopoietic elements lymphomas are composed of B-cells, T-cells or natural killer cells of varying degrees of maturity that mainly affect solid tissues or lymph nodes (2).

Cytogenetics and targeted molecular assays are now routine and necessary for the diagnosis and prognostication of most myeloid neoplasms. Molecular methods to detect specific mutations include sequencing methods (Sanger or Next-Generation Sequencing (NGS) gene panels) or modified polymerase chain reaction (PCR) techniques using genomic DNA. Most hematologic neoplasms require the analysis of chromosomal abnormalities to detect gene fusions and rearrangements by karyotype and/or fluorescence in situ hybridization (FISH) or RNA-based PCR assays may be performed, which does not require amplification of large intronic regions. After the initiation of the treatment, quantitative PCR (qPCR) or digital droplet PCR (ddPCR) assay may be used to asses for response to therapy, measured by the decrease in fusion protein transcript level. Measurable residual disease (MRD) is an important biomarker that is used for prognostic, predictive, monitoring, and efficacy-response assessments (3).

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Mature lymphoid neoplasms

These neoplasms can be classified as Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL). B-cell NHL is far more common than T-cell or NK-cell NHL and comprises approximately 80 % of lymphomas in Europe. Usually they are very heterogenous in their etiology and pathogenesis, but many are derived from the germinal center reaction (2).

Chronic lymphatic leukemia

CLL/SLL (Small lymphocytic lymphoma) is defined as a monoclonal lymphoproliferative disease characterized by the proliferation and accumulation of morphologically mature but immunologically dysfunctional B-cell lymphocytes (4).

The most frequently mutated genes in CLL are *NOTCH1* (10–15%), *SF3B1* (10%), *TP53* (5–10%), *ATM* (10–15%), and *MYD88* (3–8%) . Immunoglobulin genes are frequently rearranged and with somatic hypermutation in 50–60%. Based on the mutation status of the immunoglobulin heavy-chain variable region (*IGHV*) two subtypes of CLL were described considered as mutated *IGHV* (M-CLL) and as unmutated *IGHV* (U-CLL). U-CLL cells are associated with an aggressive disease as compared to M-CLL cells exhibit a good prognosis with low-risk genetic alterations. Cytogenetic markers are used because the acquired chromosomal abnormalities are observed in approximately 80% of individuals with CLL. Cytogenetic markers can be used to categorize patients into prognostic groups. Patients with normal cytogenetics have a median survival of approximatelly 111 months. Deletion 13q14.3 (median survival 133 months) is the most common chromosomal abnormality occurring in 40–60% of patients. Deletion 11q22.3 (median survival 79 months) is present in up to 20 % patients and 11q – patients have an aggressive clinical course characterized by a bulky lymphadenopathy and a shorter progression free survival (PFS). Deletions within the chromosome 17p13 (a median survival of 32 months) locus have been reported in 4 to 16% of the cases of CLL and show a poor survival due to an advanced disease at diagnosis, a short time to the first treatment, and a high risk of chemorefractoriness to alkylating agents and purine analogues. *TP53* mutations can be seen in the absence of deletion 17p13 in at least 20% of the cases. Trisomy 12 (a median survival of 114 months) defines a subgroup of CLL with a more frequent atypical morphology including prolymphocytes and intermediate to poor prognosis (5,6). Table 1 summarizes risk stratification in CLL.

Burkitt lymphoma (BL)

BL is an aggressive B-cell lymphoma characterized by a high degree of proliferation of the malignant cells and deregulation of the c-*myc* gene. Typically monomorphic proliferation of a medium sized transformed germinal center related B-cells is present. The diagnosis of BL is based on morphologic findings, immunophenotyping results, and cytogenetic features. BL has a characteristic chromosome abnormality of translocation at chromosome 8q24 involving *MYC* usually with chromosome 14q32 involving *IGH* (table 1). Variant translocations occur with the lambda light chain gene (*IGL*) at chromosome 22q11 or the kappa gene (*IGK*) at chromosome 2q12 in up to 16% of cases (2). However, BL can have overlapping morphologic and immunophenotypic features, and the characteristic t(8;14) translocation with diffuse large-B-cell lymphoma (DLBCL) (7). Thus, translocations involving *MYC* are characteristic but not specific for BL (2). The most frequent genetic events in pediatric BL cases up to 90% represent somatic single-nucleotide variants, insertions, and deletions (SNV/indels) of the *ID3-TCF3-CCND3* pathway. In adults BL with a significantly lower frequency of only 63% (8).

Diffuse Large B-Cell Lymphoma (DLBCL)

DLBCL represent a group of aggressive B-cell lymphomas with underlying genetic diversity and variable clinical presentations. Based on cell-of-origin several subtypes of DLBCL were identified: GCB – Germinal center B-cell, ABC – activated B-cell, PMBL – primary

mediastinal B-cell, and 15–20% of cases are unclassified. The 2016 revision of the World Health Organization (WHO) classification recognized and introduced a new entity, high-grade B-cell lymphoma (HGBCL), defined by the presence of *MYC* and *BCL2* and/or *BCL6* rearrangements and is present in approximately 8% of DLBCL (table 1). Approximately 10–15% of patients with untreated DLBCL have a rearrangaement of the *MYC* oncogene (9).

Mantle cell lymphoma (MCL)

MCL is a B-cell neoplasm characterized by the expansion of mature B cells frequently co-expressing CD5 that tend to widely spread in bone marrow, blood, lymphoid tissues, and extranodal sites. The tumor cells carry the t(11;14)(q13;q32) that leads to the constitutive overexpression of cyclin D1. Cryptic rearrangements of IG regulatory regions could be an alternative oncogenic mechanism in a minor subgroup of patients (10). *TP53* gene aberrations (mutations or deletions) are a well-established high-risk factor in MCL and were associated with an activated MYC pathway, hyperproliferation, deletion of 9p, and worse clinical prognosis (table 1). At diagnosis, the frequency of *TP53* mutations is about 11%–25%; the frequency increases to 45% at relapse. The presence of both *TP53* deletion (detected by FISH) and *TP53* mutations (detected by DNA sequencing) was associated with the worst survival. *TP53* gene mutations may co-exist with other aberrations such as *NOTCH1* mutation (71%), deletion of *CDKN2A* (del9p21) (31%), and deletion of *TP53* (del17p13) (31%). Lack of *SOX-11* with mutated *IGHV* identified a subset of MCL patients with a favorable prognosis. Patients with *IGHV* mutation may exhibit a better outcome compared to those with unmutated *IGHV*. A complex karyotype, defined as having three or more chromosomal abnormalities in addition to t(11;14), is generally considered as a high-risk factor (11).

Folicullar lymphoma (FL)

FL is considered as the most common indolent B cell lymphoma. Histologically is characterized by a follicular or nodular pattern of tumor cell growth. More than 85% of FL cases harbor the characteristic t(14;18)(q32;q21), which occurs in pro- or pre-B cells of the bone marrow. Using sensitive techniques, the t(14;18) may be detected in B cells from peripheral blood and/or lymphoid tissues of a large proportion (up to 70%) of healthy individuals although the vast majority of them will never develop FL, indicating that *BCL2* deregulation alone is insufficient for tumorigenesis. In addition to t(14;18), FL has a characteristic genomic profile, with frequent losses of 1p (15–20%), 6q (20–30%), 10q (20%), and 13q (15%), and gains of 1q (25%), 2p (25%), 8q (10%), 12q (20%), and 18q (30%), and trisomies 7 (20%), 18 (20–30%), and chromosome X (20%) (table 1) (12).

Hairy cell leukemia (HCL)

HCL is an uncommon chronic LPD characterized by progressive bone marrow failure due to infiltrating malignant B cells with “hairy-like surface projections” provoking frequent infectious complications (13). HCL comprises the clonal hematologic malignancies of classical (cHCL) and variant (vHCL). The mutations present in each HCL subtype are distinct, with *BRAF*V600E mutations in 100% of cHCLs, whereas 30% of vHCLs harbor activating mutations in *MAP2K1*, encoding the MEK1 kinase just downstream of BRAF. Studies of diverse cancers marked by the *BRAF*V600E mutation suggest that additional alterations are frequently required for tumor initiation and/or progression in *BRAF*V600E-mutant cells. Durham et al. recently detected coexistence of *CDKN1B* and *BRAF*V600E mutations in 16% of cHCLs (14).

Mucosa-associated lymphoid tissue (MALT) lymphoma

MALT lymphomas are a diverse group of lymphoid neoplasms with B-cell origin, occurring in adult patients and usually having an indolent clinical behavior. These lymphomas may arise in different anatomic locations, sharing many clinicopathological characteristics, but

also having substantial variances in the aetiology and genetic alterations. MALT lymphomas can occur at any extranodal site. The most common anatomic sites are the stomach (30%), followed by eye/adnexa (12%), skin (10%), lung (9%), and salivary gland (7%). However, these lymphomas have been described at many other mucosal organs, such as thyroid, liver, small intestine, large intestine, bladder, dura, and many other sites. Chromosomal translocations are recurrent in MALT lymphomas with different prevalence among different sites, being the 4 most common: t(11;18)(q21;q21), t(1;14)(p22;q32), t(14;18)(q32;q21), and t(3;14)(p14.1;q32). All these translocations and their products target the activation of nuclear factor k-light-chain-enhancer of activated B-cells (NF-kB) pathway. MALT lymphomas have highly altered variable heavy chain immunoglobulin (*IGHV*) and variable light chain immunoglobulin (*IGLV*) genes. Beyond translocations, a spectrum of chromosomal numerical abnormalities has been described in MALT lymphomas. The most common numerical alterations found in MALT lymphomas are trisomy of chromosome 3 or 18, although the frequencies at which these trisomies occur vary markedly with the primary site of disease (15).

Multiple myeloma (MM)

MM is the second most common hematologic malignancy. Typical clinical symptoms of MM include bone destruction, hypercalcemia, renal failure, cytopenia, and immune paralysis. Symptomatic multiple myeloma can be preceded by 2 premalignant conditions called monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma (SMM), all of which share several genetic features. Translocations can be found in half of MGUS and MM patients. Most translocations involve the IgH locus (14q32), which puts oncogenes under the influence of the powerful IgH enhancer and thus result in upregulation. Translocations involving the immunoglobulin lambda (IgL) locus are present in 10% of patients with newly diagnosed MM and up to 20% in relapsed-refractory MM and are indicative of poor prognosis (table 2) (16). The most common result of IgH translocation is dysregulation of cyclin D (*CCND*). It involves t(11;14), t(12;14), and t(6;14). In about 50% of myeloma patients, mutations induce aberrant signaling in the MAPK/ERK pathway (*NRAS*, *KRAS*, *BRAF* and *EGR1*, and *FGFR3*). About 15% of MM patients show mutations affecting DNA repair pathways like *TP53*, *ATR*, *ATM*, and *ZFHX4* genes, which are associated with a shorter survival. Moreover, in about 20% of MM patients mutations involving the NFκB pathway can be detected. Most MM cases are aneuploid, in which there are frequent gains and losses of complete chromosomes or chromosome arms (17p, 1p, 13q, and 16q). According to the ploidy status, MM is usually categorized in hyperdiploid and non-hyperdiploid MM. The hyperdiploid (H-MM) group, which accounts for 50–60% of all MM cases, is characterized by the presence of trisomies that typically affect the odd chromosomes (17).

Myeloid neoplasms

Myeloid neoplasms are clonal hematopoietic proliferations representing a wide range of clinical, hematologic, genetic, and immunophenotypic properties and with a variable rate of genetic instability and clonal progression. Based on these properties, myeloid neoplasms are divided into 10 broad categories with more than 60 entities. Based on the complete blood count data (CBC) and blood smear morphology, myeloid neoplasms can be segregated into four broad disease categories – Acute myeloid leukemia (AML), Myelodysplastic syndrome (MDS), Myeloproliferative neoplasm (MPN), and Myelodysplastic/myeloproliferative neoplasm (MDS/MPN) (18).

Myeloproliferative neoplasms (MPN)

MPNs are characterized by an excessive production of terminally differentiated blood cells that are fully functional. All MPN entities arise from a single somatically mutated hemato-

poietic stem cell (HSC) that clonally expands and gives rise to virtually all myeloid cells, and B and natural killer (NK) cells. The clonal expansion of the MPN HSC is accompanied by single or multilineage hyperplasia. Somatic mutations are responsible for the clonal expansion of HSCs not only in MPNs, but also in most types of myeloid malignancies (19). Among MPNs, chronic myeloid leukemia is characterized by the presence of Philadelphia chromosome (Ph) resulting from the translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)] leading to BCR/ABL1 gene fusion. The Ph-negative MPNs encompass 3 clinical subtypes: polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). In contrast to chronic myeloid leukemia, disease-specific genetic abnormalities have not been detected that distinguish PV, ET, and PMF (20).

Chronic myeloid leukemia (CML)

The Philadelphia chromosome, originating from a balanced reciprocal translocation *t* (9; 22) (*q*34; *q*11), is present in more than 90% cases of CML. This translocation causes the fusion of the Abelson murine leukemia (*ABL*) proto-oncogene on chromosome 9 with the interrupted end of the breakpoint cluster region (*BCR*) of chromosome 22. The chimeric gene encodes a protein with a high tyrosine kinase activity which acts as a tumorigenic factor. These alterations results in an excessive production of granulocytes in the bone marrow causing both splenomegaly and hyperleukocytosis. In the course of CML progression, additional chromosomal abnormalities appear in particular during accelerated and blastic phase and can cause genetic instability. These abnormalities which are found in the Ph+ cells are classified into major and minor. Major pathway additional abnormalities include trisomy 8, additional Ph derivation (+der (22) *t* (9; 22), isochromosome 17 (*i* (17) (*q*10)), trisomy 19, and others. Minor pathway additional abnormalities is less common and not sufficiently studied. It includes aneuploidies −7, −17, +17, +21, and −Y and one balanced structural abnormality *t* (3; 21) (*q*26; *q*22). In patients with Ph− cells, additional abnormalities found could be a reciprocal translocation *t* (6; 9) (*p*21; *q*34.1), a chromosomal marker (+mar), a trisomy 8, and others. On the other hand, the absence of the Ph chromosome and the presence of −7 (monosomy 7) contribute to the evolution towards a myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) (21). The main group of treatment for CML consists of tyrosine kinase inhibitors (TKI), which represent targeted therapy. BCR-ABL kinase is present only in leukemic cells and its complete blockade by TKI leads to apoptosis. The unique genetic feature of the BCR-ABL gene allows the quantification of the treatment response by quantifying the expression of the fusion gene and analyzing the number of Ph chromosome-positive metaphases. Molecular response is assessed by qPCR based on assessment of the ratio of BCR-ABL to a control gene, most commonly ABL. If the BCR-ABL/ABL ratio is ≤ 0.1% International Scale (IS) (≥ 3 log reduction of BCR-ABL transcript) patient has achieved a major molecular response (MMR).

MR4 (≥ 4 log reduction; ≤ 0.01%)

MR4,5 (≥ 4 ,5 log reduction; ≤ 0.0032%)

MR5 (≥ 5 log reduction; ≤ 0.001%).

It is therefore important at the time of diagnosis to carry out an examination to determine the presence and determination of the amount of transcripts.

Ph- negative myeloproliferative neoplasms

A major characteristic of Ph-negative MPNs is an increased signaling through the Janus kinase (JAK) signal transducer and activator of transcription (STAT) pathway as well as through the phosphatidylinositol 3-kinase (PI3K)-AKT (also known as protein kinase B) pathway in erythroid and myeloid cells. The most significant evidence of molecular pathology was reported in 2005 with the identification of the somatic mutation *JAK2*-V617F. This mutation in *JAK2* exon 14 gene occurs in approximately 95% of patients with PV and about 60% of those with PMF and ET.

Somatic activating mutations in the MPL virus oncogene *(MPL)* were identified in patients with *JAK2*-nonmutated ET and PMF but not in patients with PV. The *MPL* gene is located on chromosome 1p34, encodes the thrombopoietin receptor and is a key factor for growth and survival of megakaryocytes. Acquired mutations at codon W515 constitutively activate the thrombopoietin receptor by cytokine-independent activation of the downstream JAK-STAT pathway. Recurrent pathogenic mutations include the common W515L and W515K and the rare W515A, W515R and W515S mutations. The 2 most recurrent mutations W515L and W515K are found in approximately 15% of *JAK2*-V617F-nonmutated MPN that is 5% of ET and up to 10% of PMF. Alternative mutations have also been reported in rare cases including V501A, S505C, A506T, V507I, G509C, L510P, R514K, and R519T, although the pathogenic significance of some of these mutations is not clear. The median overall survival of patients was approximately 9 years in both *MPL*-mutated and *JAK2*-mutated PMF (20).

Mutations in calreticulin (*CALR*) are also found in approximately 25–35% of patients with ET and 35–40% of those with MF (21). *CALR* is not known to have a direct role in cytokine signaling, hematopoiesis, or cell fate decisions, and therefore the mechanism(s) by which *CALR* mutations result in megakaryocytic proliferation and an ET/MF phenotype were not initially apparent (22). It is clear that the primary mechanism of *CALR*-driven transformation lies in its interaction with MPL, which triggers JAK2-dependent signaling pathways, although it remains to be seen whether mutant *CALR* may also act through other pathways, such as Ca^{2+} signaling or transcriptional regulation (table3) (23).

Myelodysplastic syndrome (MDS)

MDS are a heterogeneous group of hematopoietic precursor cell diseases with altered cell proliferation and maturation characterized by peripheral cytopenia due to ineffective hematopoiesis, dysplasia of one or more cell lineages, and an increased risk of transformation to acute myeloid leukemia (AML). The hallmark of MDS is bone marrow failure due to the growth of somatically mutated clonal hematopoietic stem cells (24). Karyotypic abnormalities are seen in approximately 30–50% of patients with MDS and correlated with prognosis. The most frequent cytogenetic abnormality in MDS is deletion 5q with frequency about 15%. Deletion 5q32-33 is frequently associated with 5q- syndrome and patients with this syndrome have a better overall survival and less risk of transformation to AML. But deletion 5q31is typically present in MDS that arose in connection with previous chemotherapy and has a more aggressive course with a high risk of progression to AML. Monosomy or deletion 7 is connected with a poor prognosis. Approximately 10% of MDS patients have an abnormality of the chromosome 7 either alone or as part of a complex karyotype. Abnormalities of the chromosome 7 occur in up to 50% of patients with MDS arising after the treatment with alkylating agents. Chromosome 7 abnormalities associated with 5q- or transcription factor *RUNX1* mutation are more frequent in these patients compared to other MDS patients, which points to a multistep process of MDS development. Other chromosomal abnormalities such as trisomy 8 and deletion 20 are frequently present in MDS patients. On the other hand, mutations are detectable by next generation sequencing (NGS) in more than 80% of patients with MDS with distinct mutation profiles observed in different MDS subtypes – *TP53, EZH2, ETV6, RUNX1, ASXL1* present in 3–14 % MDS patients connected with a poor prognosis. The mutations of genes that regulate mRNA splicing (*SF3B1,ZRSR2,ZRSF2*) occur in 45-85% of MDS patients. Their result is the synthesis of disturbed proteins, which are involved in the pathogenesis of MDS. With increasing knowledge and new diagnostic possibilities, primarily in the field of genetics, a revised international prognostic scoring system (IPPS-R) was created (table 4). The basis is cytogenetics, percentage representation of blasts and cytopenias (25).

Acute myeloid leukemias (AML)

AML is a heterogenous group of hematopoietic malignancies characterized by a proliferation of immature cells (blasts). Early classification systems were based on the morphologic features of these blasts, while in last two decades, the predominant classification systems by the World Health Organization (WHO) have increasingly incorporated immunophenotypic and genetic characteristics to refine these groupings. The 5th edition of the WHO classification evaluates three categories based on – (1) reccurent genetic abnormalities, (2) myelodysplasia-associated genetic changes, and (3) germline predisposition (26,27).

AML is a lethal disease and has a 5-year relative survival rate of 24.2%. However, the outcomes are heterogenous and the overall survival rates range from 5% to 70%. Thus, a need exists for prognostive markers to predict outcomes and guide therapeutic decision-making. The strongest prognostic factor for predicting therapeutic response and survival is cytogenetic subgrouping. Risk groups in AML were classified into three cathegories according to the 2022 European Leukemia Net (ELN) risk stratification based on genetics (Table 5) (28). Translocations t(15;17), t(8;21), inv16/ t(16;16), a normal karyotype and mutated *NPM*, or a normal karyotype with biallelic *CEBPA* mutations present favorable risk with a 5-year survival rate of 50-80%. But aberations like MLL, inv3, t(6;9), -7/del(7q), -5/del5q, *TP53* deletions and a complex karyotype are of adverse risk with an overall survival rate of 5-20%.

Molecular testing for t(15;17), *FLT3, NPM,* and *CEBPA* is informative and has therapeutic implications. Translocation between choromosomes 15 and 17 involves the *PML* gene and the retinoic acid receptor gene (*RARA*). The resulting fusion protein, PML-RAR-α is oncogene that is typical for acute promyleocytic leukemia (APL). Identification of this genetic alteration led to development of the therapy that specifically target aberrant cells. All-trans retinoic acid (ATRA) binds to fusion protein inside cells and blocks its function. In *FLT3*-mutated AML, midostaurin is added to intensive chemotherapy. Menin inhibitors are being evaluated as a treatment option for patients with *KMT2A* rearrangements or *NMP1* mutations (2, 26).

CONCLUSION

Most hematologic malignancies are clonal neoplasms and have specific somatic genetic and molecular characteristic which may influence therapeutic response and prognosis. The molecular basis of each tumor type is becoming essential to the diagnosis, in addition to determining therapy and prognosis. Understanding of the molecular pathogenesis of the diseases has improved, new therapeutic approaches have become crucial. Personalized therapeutic approaches assume prominence, emphasizing the need for tailored interventions based on individual patient characteristics. Incorporating cytogenetic changes alongise othe prognostic factors becomes crucial in determining the optimal treatment strategy. With expanding and increasing use of NGS panels, not just for detection of diagnostic gene mutations but also for detection of clonal lymfoid populations, chromosomal fusions, or minimal residual disease.

Table 1 Recurrent mutations and genetic alterations in mature lymphoid neoplasms

BNHL	Molecular alterations	Risk stratification
B-CLL/SLL	del 13q, 11q, 17p13, 6q21, trisomy 12	**Very high-risk disease:** 17p deletion and/or *TP53* mutations **High-risk disease:** *IGHV* unmutated (without 17p deletion and *TP53* mutation) **Standard-risk disease:** *IGHV* mutated (without 17p deletion and *TP53* mutation) (5,6)
Burkitt lymphoma	translocation at 8q24 (*MYC*) with 14q32(*IGH*); translocations at 22q11(*IGL*) or 2p12 (*IGK*)	*MYC*_translocation, including deletion of 13q, a gain of 7q, or complex cytogenetics may portend a worse prognosis Double hit mutations in *ID3*, *CCND3*, and mutations in 18q21 CN-LOH indicate a poor response to therapy and poor prognosis (29)
DLBCL	rearrangements *of IGH, IGK, IGL,MYC*, 3q27, t(14;18)	16q22-q24, 6p21-p25, 12q22-q24, 11q23-q25, 19q13, 1q21-q23, 8q24, and 19p13, and -17 appeared to be associated with a worse prognosis (30)
Follicular lymphoma	t(14;18), abnormalities *BCL6* and 3q27	deletions of 1p, 6q, and 17p, and gains of 7 and 12q are strongly associated with a poor prognosis also correlate with a higher risk of transformation (31)
Hairy cell leukemia	*BRAF* V600E	
MALT lymphoma	t(11;18), t(14;18)(q32;q21), t(3;14)	
Mantle cell lymphoma	t(11;14)(q13;q32)	**Cluster C1—Best prognosis** mutated IGHV, *CCND1* and *TP53*, amplification of 11q13, and active BCR signaling ***Cluster C2***—deletion of 11q, *ATM* mutations, upregulated TNF-α, NF-kB, and DNA repair pathways ***Cluster C3***—mutations in *NOTCH1*, *NSD2*, *SP140*, and *KMT2D*; amplification of 13q; deletion of 6q; and downregulated TNF-α, NF-kB, BCR signaling, and MYC target pathways ***Cluster C4*—Worst prognosis** deletion of 13q, 17p/*TP53*, and 9p; *TP53* mutations, complex copy number abnormalities; upregulated MYC pathways (11)

Abbreviations: del – deletion, DLBCL – diffuse large B-cell lymphoma, IGH – immunoglobulin heavy chain, IGK – kappa gene, IGL – light chain gene, t – translocation

Table 2 Recurrent mutations and genetic alterations in multiple myeloma (16, 17).

Genetic abnormalities	Affected genes	Frequency	Prognosis
t(4;14)	*FGFR3, MMSET*	11–15%	High risk
t(6;14)	*CCND3*	1–2%	Standard risk
t(11;14)	*CCND1*	15%	Intedmerdiate risk
t(14;16)	*MAF*	3–5%	High risk
t(14;20)	*MAFB*	1%	High risk
Del 1q	*FAM46C, CDC14A, MTF2, CDKN2C*	30%	Shorter survival
Gain of 1q	*CKS1B, MUC1, MCL1, ANP32E, BCL9, PSMD, PDZK1*	50% NDMM	Poor prognosis
Del 13q	*RB1*	45%	del(13q) – independent favorable impact on OS monosomy 13 – shorter OS
Del 17p	*TP53*	5–12% NDMM	Shorter survival
KRAS		~ 50%	Neutral
NRAS		~ 50%	Worse outcome
BRAF		~ 50%	Negative influence on survival
EGR1		~ 50%	Favorable effect on outcomes

Abbreviations: del – deletion, NDMM - newly diagnosed MM; OS – overall survival; t - translocation

Table 3 Recurrent mutations and genetic alterations in Ph negative myeloproliferative neoplasms (19)

Gene	Location	Mutation	Protein function	Frequency	Consequence
JAK2	9p24	*JAK2*V617F	tyrosine kinase associated with cytokine receptors	95% PV 50–60% PMF 50–60% ET	increased RBC, WBC, PLT production
		JAK2 exon 12		3% PV	
MPL	1p34	MPL515L/K/A/R MPLS505N	TPOR	2–3% ET	increased PLT production
		other missense mutations		3–5% PMF	
CALR	19p13	indel exon 9	Mutant: activator MPL	20–25% ET 25–30% PMF	increased PLT production

Abbreviations: RBC – red blood cells, ET – Essential thrombocytemia, PLT – platelets, PMF – Primary myelofibrosis, PV – Polycytemia vera, TPOR – trombopoietin receptor, WBC – white blood cells

Table 4 Recurrent mutations and genetic alterations in Myelodysplastic syndrome (24,25)

Cytogenetic alterations	Risk category
del(11q), -Y	Very good
del(5q), del(12p), del(20q), double including del(5q), normal karyotype	Good
del(7q), + 8, + 19, isochromosome i(17q), any other single or double independent clones	Intermediate
-7, inv(3)/t(3q)/del(3q), double including – 7/del(7q), complex karyotype: > 3 alterations	Poor
complex karyotype: > 3 alterations	Very poor
Biallelic *TP53*	Very poor
SF3B1	Favorable risk
EZH2, ASXL1	Lower risk
RUNX1, NRAS	Associated with chromosome 7 abnormalities

Abbreviations: del – deletion, inv – inversion, t – translocation

Table 5 Risk groups in AML according to the European Leukemia Net risk stratification

Favorable risk	Intermediate risk	Adverse risk
t(8;21)(q22;q22.1)/ *RUNX1::RUNX1T1*	*FLT-ITD* (regardless of allelic ratio or *NPM1* mutation	t(6;9)(p23;q34.1)/ *DEK::NUP214*
inv(16)(p13.1;q22)	t(9;11)(p21.3;q23.3)/ *MLLT3::KMT2A*	t(v;11q23.3/*KMT2A* rearranged
t(16;16)(p13.1;q22)/ *CBFB::MYH1*	cytogenetic and/or molecular abnormalities not classified as favorable or adverse	t(9;22)(q34.1;q11.2)/ *BCR::ABL1*
mutated *NPM1* without *FLT3-ITD*		(8;16)(p11;p13)/ *KAT6A:CREBBP*
bZIP in-frame mutated *CEBPA*		inv(3)(q21.3;q26.2) or t(3;3)(q21.3;q26.2)/ *GATA2,MECOM (EVI1)*
		t(3q26.2;v) *MECOM (EVI1)* rearranged
		monosomy 5 or del(5q)
		monosomy 7
		monosomy 17/abn(17p)
		complex karyotype > 3 unrelated chromosomal abnormalities
		mutated *ASXL1*, *BCOR*, *EZH2*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1* or *ZRSF2*
		mutated *TP53* (variant allele frequency ≥ 10%)

(26)

Abbreviations: abn – abnormal, del – deletion, inv – inversion, t – translocation

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METABOLIC FLEXIBILITY: TARGETING MITOCHONDRIAL DYNAMICS IN CANCER THERAPY

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Abstract

Various types of cancer exhibit distinct sensitivities to anticancer drugs, reflecting their unique developmental pathways. Exploiting mitochondrial dysfunction in cancer cells presents novel therapeutic opportunities. In recent years, advancements have underscored the significance of oxidative phosphorylation, fatty acid oxidation, and glutamine addiction in cancer cells, shifting attention towards mitochondrial metabolism. The foundation of mitochondrial medicine lies in comprehending targeted mitochondrial therapy and the development of specific mitochondrial drugs. Certain alterations within mitochondrial metabolism lead to the accumulation of oncometabolites, serving as epigenetic regulators, or an increase in reactive oxygen species production, both of which contribute to tumorigenesis. Importantly, the substantial involvement of mitochondrial metabolism in tumorigenesis offers potential avenues for exploiting as strategies for cancer therapy. Integrated pharmacological approaches targeting mitochondrial metabolic pathways are imperative for an effective eradication of tumor masses and a concurrent elimination of small subpopulations of mitochondria. The significance of mitochondria in cancer is undeniable, offering numerous appealing targets for both tailored and personalized cancer therapy.

Key words: cancer, mitochondria, oxidative phosphorylation, tricarboxylic acid cycle

INTRODUCTION

Tumor cells possess the capacity to adapt their bioenergetic profile in response to microenvironmental conditions and nutrient availability. This metabolic plasticity enables the subpopulations of tumor cells to dynamically adapt metabolic processes to the demands of energy generation and biosynthetic requirements. The intratumoral cellular heterogeneity is characterized by notable alterations in metabolic profiles, dependent upon cell type (1). This phenomenon can facilitate tumor cell survival under altered microenvironmental conditions, as well as during and after chemotherapy (2). Each type of cancer exhibits distinct sensitivities to administered anticancer drugs, partially reflecting variations in mitochondrial pathways such as oxidative phosphorylation, fatty acid metabolism, glutamine metabolism, or one-carbon metabolism. These alterations arise from mutations in oncogenes, tumor suppressor genes, and metabolic enzymes (3). Consequently, a metabolic

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reprogramming occurs, facilitating a rapid cell proliferation and the generation of reactive oxygen species, which cancer cells exploit to sustain pro-tumorigenic signaling pathways and evade cell death. Reprogramming of mitochondrial metabolism in cancer presents appealing targets for therapy (1). Despite the challenges inherent in targeting mitochondrial metabolism, several inhibitors of key enzymes are undergoing clinical trials (4–6). Combination strategies and novel drugs targeting metabolic pathways hold a promise for advancing precision medicine in cancer.

Mitochondria and Cancer

The principal mitochondrial metabolic pathways encompass the tricarboxylic acid (TCA) cycle, fatty acid oxidation (FAO), the electron transport chain (ETC), and oxidative phosphorylation (OXPHOS), collectively engaged in the catabolism of biomolecules and energy generation. Furthermore, mitochondria serve as a source of precursors for numerous biomolecules and adjust to varying metabolic conditions through alterations in nuclear transcription (7). The involvement of damaged mitochondria in initiating the Warburg effect across diverse cancer types, characterized by a uniform decrease in OXPHOS, has been firmly established. Notable examples include oncocytic tumors, neuroblastomas, renal cell carcinomas, and astrocytic brain tumors. However, certain cancers exhibiting the Warburg metabolic phenotype preserve intact mitochondrial respiration, including leukemia, lymphoma, pancreatic ductal carcinomas, melanomas of the high OXPHOS subtype, and endometrial carcinoma (8). Furthermore, certain cancer subtypes not only maintain functional mitochondria but also rely on mitochondrial respiration for essential cellular processes. Consequently, these cancers exhibit a sensitivity to the inhibition of OXPHOS (8). In this context, it is firmly established that mitochondrial energy pathways undergo alterations in the regulation of both glycolysis and mitochondrial respiration. Cancer cells are compelled to foster metabolic plasticity to adapt their metabolic processes to the demands of energy generation and biosynthetic requirements (9). The pivotal role of mitochondria in tumorigenesis is unsurprising, given their significant involvement in various aspects of cancer development. They are central to several classical hallmarks of cancer, including metabolic reprogramming, sustained proliferation, promotion of tumor-associated inflammation, evasion of cell death mechanisms, facilitation of invasion, and stimulation of angiogenesis (10).

To counterbalance the 18-fold difference in efficiency, glycolysis is stimulated through the upregulation of glucose transporters, notably GLUT1, to enhance glucose uptake. Additionally, an overexpression of key enzymes in glycolysis, such as hexokinase-2 and lactate dehydrogenase, contributes to this activation. The heightened glycolytic flux leads to the accumulation of glycolytic intermediates, which serve as precursors for various biosynthetic pathways essential for cellular proliferation. These intermediates fuel the pentose phosphate pathway, facilitating ribose production, and cytosolic nicotinamide adenine dinucleotide phosphate (NADPH) generation, crucial for nucleotide and antioxidant synthesis. Moreover, they contribute to one-carbon metabolism, necessary for mitochondrial NADPH production, methylation processes, and nucleotide synthesis (11).

Targets within the TCA cycle

The TCA cycle serves as a source of intermediates required for the synthesis of lipids, proteins, and nucleotides. To maintain the functionality of the TCA cycle, these intermediates must be replenished through a process known as anaplerosis. Two primary anaplerotic pathways have been identified: glutaminolysis (12), which produces α-ketoglutarate from glutamine, and pyruvate carboxylation, which generates oxaloacetate from pyruvate derived from glucose (13).

Various therapeutic strategies targeting the TCA cycle for cancer treatment have been under investigation. The inhibition of the mitochondrial pyruvate transporter with UK5099 attenuates OXPHOS. Perturbations in TCA cycle enzymes lead to the production of oncometabolites such as 2-hydroxyglutarate (2-HG), fumarate, and succinate, implicated in tumorigenesis.

Inhibitors targeting these enzymes include AGI-5198, AG-221, and AG-881 for isocitrate dehydrogenase (IDH), and CPI-613 for α-ketoglutarate dehydrogenase complex (14). Although challenging to target, loss-of-function mutations of the fumarate hydratase (FH) or succinate dehydrogenase (SDH) enzymes have seen success with small compounds inhibiting enzymes with a gain of function. AGI-5198 inhibits mutant IDH, resulting in a reduced 2-HG formation and an induction of glioma cell differentiation (15). Enasidenib (AG-221) and vorasidenib (AG881) are in clinical trials for acute myelogenous leukemia carrying IDH2 or IDH1/2 mutations, respectively (4,6). Additionally, devimistat (CPI-613), targeting α-KG dehydrogenase complex and pyruvate dehydrogenase, is in phase I/II trials for leukemias, lymphomas, and small cell lung cancer (5,16). The elevated levels of glutamate dehydrogenase (GDH) play a contributory role in augmenting fumarate levels, which subsequently bind to and activate the glutathione peroxidase enzyme, thereby enhancing ROS detoxification in myeloma, leukemia, breast, and lung cancer cell lines. Glioblastoma cells demonstrate a significant reliance on GDH (17). Furthermore, GDH plays a crucial role in breast cancer cells by facilitating ammonia recycling, thereby meeting the heightened demand for amino acid synthesis (18). Inhibitors of GDH include epigallocatechin-3-gallate, R162, hexachlorophene, and bithionol have been identified (14).

Numerous pharmaceuticals, encompassing inhibitors of the ETC elicit reactive oxygen species production via diverse mechanisms. Notably, enhanced ROS-induced apoptosis has been observed in cancer cells subsequent to the depletion of ATP resulting from the manipulation of glycolytic enzymes, chemotherapy, or radiation therapy. These findings underscore the potential pivotal role of ROS modulation in the context of anticancer combinatorial therapies. Moreover, recent advancements in ROS-inducing drugs have focused on achieving the fundamental objective of therapeutic selectivity in cancer treatment. Consequently, assessing the baseline ROS levels within a tumor holds a potential utility in evaluating the responsiveness to ROS-inducing agents. This assessment may be complemented by the concurrent administration of inhibitors targeting compensatory mechanisms, such as glycolysis or antioxidant proteins, enhancing the therapeutic strategy's efficacy. Inhibitors targeting the glutamine pathway disrupt glutathione formation, thereby perturbing the antioxidant system. Inhibition of the glutathione system can be accomplished with agents such as NOV-002, L-buthionine-S, R-sulfoximine, canfosfamide, or ezatiostat hydrochloride (19).

Targets within the oxidative phosphorylation

The efficacy of inhibiting OXPHOS as a targeted therapeutic strategy for cancers that depend on OXPHOS has been elucidated in multiple studies. These malignancies comprise diffuse large B-cell lymphoma (20), breast cancer (21), pancreatic ductal adenocarcinoma (22), melanoma (23), and glioma (24). However, the impact of OXPHOS on cancer drug resistance is intricate and influenced by cell types within the tumor microenvironment. Cancer cells universally bolster OXPHOS activity through various signaling pathways, which is essential for conferring the resistance to cancer therapy (25). Nicotinamide adenine dinucleotide and flavin adenine dinucleotide generated in the TCA cycle donate electrons to complexes I and II of the ETC, producing the energy for proton translocation across the inner mitochondrial membrane and ATP synthesis. Mutations in genes encoding enzymes SDH, FH, and IDH cause an abnormal accumulation of oncometabolites, resulting in a deregulation of signaling promoting cancer progression. SDH catalyzes oxidation from succinate to fumarate. It also contributes as part of Complex II of the ETC, reducing ubiquinone to ubiquinol (14). In the absence of oxygen reduction, cells accumulate ubiquinol, leading to the reversal of the SDH complex, facilitating the deposition of electrons onto fumarate. Upon the inhibition of oxygen reduction, the reduction of fumarate supports the activities of dihydroorotate dehydrogenase and complex I. Consequently, under hypoxic conditions, fumarate serves as a terminal electron acceptor in the mammalian electron transport chain to support of crucial mitochondrial functions and that ability is tissue-specific (26).

Previous data indicate a lack of anticancer activity of mitochondrial inhibitors, including metformin or ME-344 (a mitochondrial CI inhibitor), either alone or in combination. While rotenone and methyl-4-phenylpyridinium are known to inhibit Complex I, they exhibit neurotoxic effects. Conversely, deguelin, an analogue of rotenone, shows a promise as a potential chemotherapeutic drug. This scenario underscores the necessity of gaining a deeper insight into the metabolic context in which mitochondrial inhibitors may exert their anticancer effects (27). The inhibition of complex I by tamoxifen increases hydrogen peroxide production. IACS-010759, a promising novel inhibitor targeting Complex I, is undergoing clinical trials (not posted results) for the treatment of acute myeloid leukemia and specific types of solid tumors (28). Several experimental inhibitors targeting Complex II include malonate, nitropropionic acid, thenoyltrifluoroacetone, troglitazone, 3-bromopyruvate, and α-tocopheryl succinate (Fig. 1) (29).

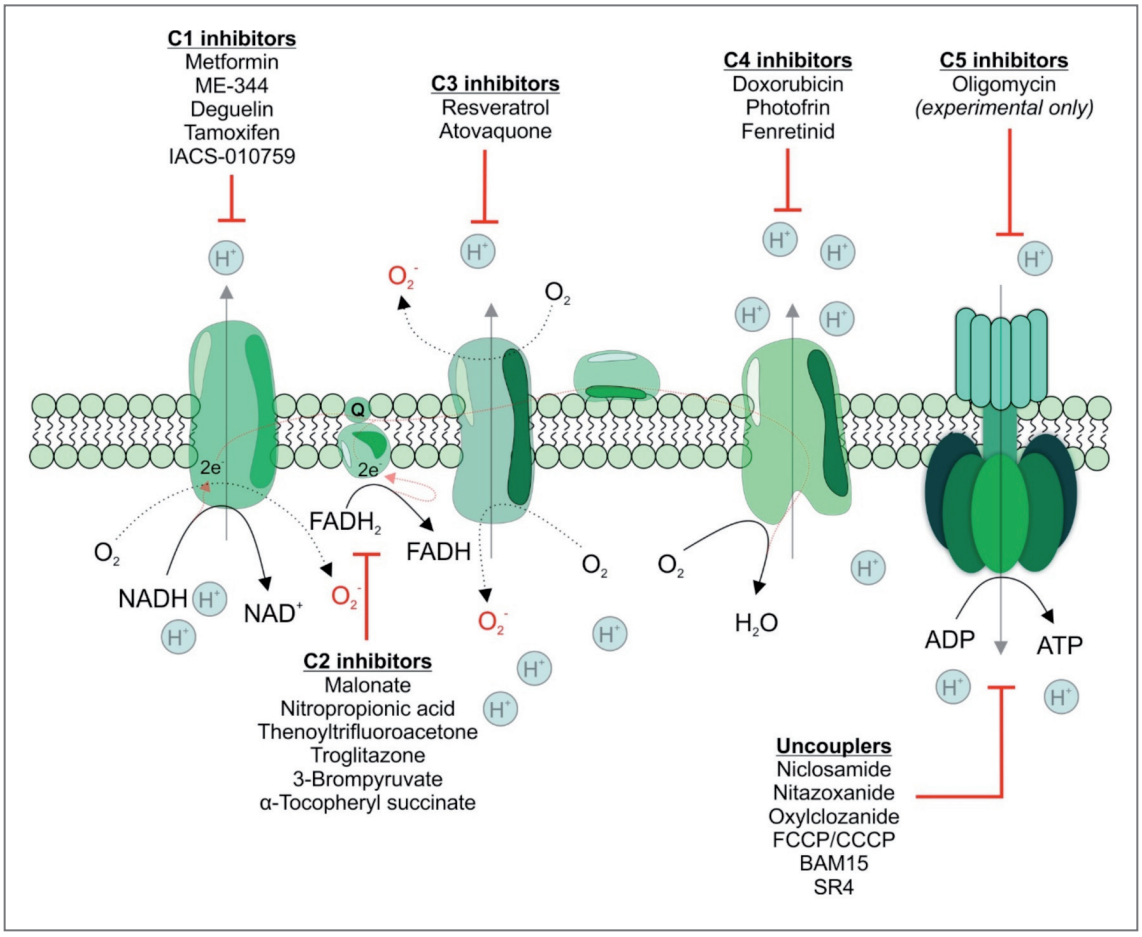

Fig. 1 Inhibiting oxidative phosporylation as a therapeutic strategy in cancer (complex – C)

Antimycin A is employed in experimental research to inhibit Complex III, whereas resveratrol has been tested for various types of cancer. Atovaquone, on the other hand, is currently being investigated in clinical trials for non-small cell lung cancer (NSCLC), particularly in combination with chemotherapeutic drugs (30). Doxorubicin, a DNA intercalating chemo-

therapeutic agent, and the porphyrin photosensitizer photofrin, sanctioned for esophageal cancer and NSCLC, both possess the capacity to inhibit Complex IV. Meanwhile, fenretinid (N-(4-Hydroxyphenyl) retinamide) is tested for various tumor types, such as ovarian cancer, B-cell non-Hodgkin lymphoma, and breast cancer (31) . To date, no promising inhibitors have been reported for Complex V, with oligomycin being the only option available, albeit primarily suitable for experimental purposes (29). Utilizing mitochondrial uncouplers presents an alternative strategy to disrupt ETC function. Compounds such as niclosamide, nitazoxanide, oxyclozanide, FCCP/CCCP, BAM15, or SR4 achieve this by facilitating proton transport across the IMM, thereby short-circuiting ATP synthesis (Fig. 1). Niclosamide finished phase I/II clinical trials for prostate and colon cancer (so far not posted results), while nitazoxanide is in phase II trials for various forms of advanced cancers (32).

CONCLUSION

It is very important to delineate the scenarios in which mitochondrial respiration becomes necessary, making the treatment of oncologic diseases with mitochondrial inhibitors very effective. A remarkable fact about targeted mitochondrial therapy in cancer is its promising potential to address tumor heterogeneity and resistance mechanisms. By specifically targeting the metabolic vulnerability of cancer cells, such therapies aim to disrupt critical pathways involved in tumor growth and survival, thereby offering novel approaches to cancer treatment.

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