Review

**GSDME** and its role in cancer: from behind the scenes to the front of the stage

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**KEY WORDS**

Gasdermin E; biomarker; detection; prognosis; tumor suppressor

**ABBREVIATIONS USED IN THIS ARTICLE**

AUC, area under the curve; CAR, chimeric antigen receptor; C-GSDM, gasdermin C-terminal domain; ChIP, chromatin immunoprecipitation; CIMP, CpG island methylator phenotype; ctDNA, circulating tumor DNA; cyt c, cytochrome c; DAMPs, damage associated molecular patterns; DFNA5, deafness, autosomal dominant 5; ER, estrogen receptor; GSDM, gasdermin; GSDMA, gasdermin A; GSDMB, gasdermin B; GSDMC, gasdermin C; GSDMD, gasdermin D; GSDME, gasdermin E; ICERE, inversely correlated with estrogen receptor; IL-1β, interleukin-1β; LDH, lactate dehydrogenase; MOMP, mitochondrial outer membrane permeabilization; N-GSDM, gasdermin N-terminal domain; PJVK, Pejvakin; Plk1, Polo kinase 1; PR, progesteron receptor; TCGA, The cancer genome atlas; Thr, threonine; TNM, tumor-node-metastasis;
ABSTRACT

Gasdermin E (GSDME), a gene originally involved in hereditary hearing loss, has been associated with several types of cancer in the last two decades. Recently, GSDME was identified as a pore forming molecule which is activated following caspase-3-mediated cleavage resulting in so-called secondary necrosis following apoptotic cell death, or in primary necrotic cell death without an apoptotic phase, so-called pyroptosis-like. This implication in cell death execution suggests its potential role as a tumor suppressor. GSDME also exhibited a cancer type-specific differential methylation pattern between tumor tissues and normal cells, implying GSDME gene methylation both as a pan-cancer and cancer-type specific detection biomarker. A bit paradoxically, GSDME protein expression is considered to be less suited as biomarker, and although its ablation does not protect the cell against eventual cell death, its protein expression might still operate in tumor immunogenicity due to its capacity to induce (secondary) necrotic cell death which has enhanced immunogenic properties. Additionally, GSDME gene expression has been shown to be associated with favorable prognosis following chemotherapy, and could therefore be a potential predictive biomarker. We provide an overview of the different associations between GSDME gene methylation, gene expression and tumorigenesis, and explore their potential use in the clinic. Our review only focuses on GSDME and summarizes the current knowledge and most recent advances on GSDME’s role in cancer formation, its potential as a biomarker in cancer and on its promising role in immunotherapies and anti-tumor immune response.

INTRODUCTION

The gasdermin E (GSDME) gene, also known as deafness, autosomal dominant 5 (DFNA5), was identified in 1998 on chromosome 7p15.3 in patients with a specific form of autosomal dominant, progressive, sensorineural and non-syndromic hearing loss. Remarkably, although the identified GSDME mutations in families with hearing loss are distinct at DNA level, they all result in skipping of exon 8 and truncation of the protein. GSDME belongs to the gasdermin (GSDM) family, which owes its nomenclature to its high expression pattern along the gastrointestinal tract and skin (dermis). In addition, expression of GSDME is reported in all vital organs. Until now, six GSDM genes have been identified in humans: GSDMA, GSDMB, GSDMC, GSDMD, GSDME and Pejvakin (PJVK). Except for PJVK, all GSDM proteins consist of a conserved N- and C-terminal globular domain, separated by a flexible hinge region. Recently, the N-terminal (N-GSDM) domain of GSDMA, -D and -E was shown to execute cell death by pore formation, and this function is
apparently inhibited by the C-terminal domain (C-GSDM) in the full length protein. In case of hearing loss, it is hypothesized that truncation of C-GSDME by skipping of exon 8, represents a gain-of-function mutation that unleashes the intrinsic pore forming activity and might result in increased death of terminally differentiated cochlear hair cells or other cells important for hearing\textsuperscript{3,4,16,18,19} (Figure 1).

Next to hearing loss, \textit{GSDME} has been associated with cancer\textsuperscript{15,16,20–40}. Genomic methylation screens unveiled \textit{GSDME} as a possible tumor suppressor gene\textsuperscript{35,37,38}. In general, methylation of promotor CpGs, frequently associated with transcriptional silencing, may serve as a mechanism to inactivate tumor suppressor genes in cancer\textsuperscript{41,42}. In that respect it was hypothesized that DNA promoter methylation of \textit{GSDME} prevents GSDME-mediated regulated cell death and in that way contributes to tumorigenesis (Figure 1). However, recent breakthroughs on the function of the \textit{GSDM} gene family shed new light on the role of GSDME in cell death and consequently on its contribution to tumor biology. In this review we first focus on the biological function of GSDME in order to understand the current associations between GSDME and cancer. Next, we evaluate \textit{GSDME} methylation/expression as a detection, prognostic and predictive cancer biomarker. Finally, the effect of \textit{GSDME} protein expression on chemotherapeutic treatment will be explored. Overall we conclude that \textit{GSDME} methylation and expression may have substantial clinical utility as diagnostic and prognostic marker, and even as a therapeutic target during chemotherapy-mediated cell death.

\section*{FUNCTION OF GSDME}

\textbf{GSDME executes necrotic cell death by pore formation}

The physiological function of GSDMs was unknown for decades, despite their association with different diseases such as alopecia\textsuperscript{43,44}, asthma\textsuperscript{45–48}, hearing loss\textsuperscript{1,49} and cancer\textsuperscript{13,50–55}. The conserved N-terminal domain of all GSDMs but PJVK is shown to execute necrotic cell death\textsuperscript{17}. Under physiologically normal conditions, this cytotoxic function is impeded by C-GSDM\textsuperscript{16,56}. Depending on the cell death trigger, GSDMs are activated by proteolytic cleavage by different proteases, thereby liberating N-GSDM\textsuperscript{33,46,56–62}. GSDME is cleaved by the crucial apoptotic executioner caspase-3\textsuperscript{33,59} (Figure 2). In essence, apoptosis is a containment program preparing the dead cell corpse to be removed by phagocytosis\textsuperscript{63}. It is morphologically characterized by plasma membrane blebbing and the release of apoptotic bodies that contain cellular material (Table 1). Usually, apoptotic cells are cleared by neighboring phagocytes before they lose membrane integrity. When phagocytes are absent, the contained apoptotic cells progress to a necrotic cell death modality associated with swelling and plasma membrane permeabilization, termed ‘secondary necrosis’ (Table 1)\textsuperscript{64}. In bone marrow derived macrophages, induction of the mitochondrial apoptotic pathway by overexpressing Bax results, among others, in caspase-3-mediated cleavage of GSDME and secondary necrosis following apoptotic plasma membrane blebbing (Figure 2)\textsuperscript{99}. When the \textit{GSDME} gene is ablated, the necrotic morphology
of late apoptotic cells remains absent and the cells remain for longer time in the apoptotic phase characterized by membrane blebbing and containment of the plasma membrane, suggesting that GSDME is responsible for the necrotic plasma membrane permeabilization and dispersion of cellular content in the environment. Moreover, N-GSDME apparently targets mitochondria and facilitates the release of cytochrome c (cyt c)\textsuperscript{65}, thereby creating a self-amplifying feed-forward loop during apoptosis by the consecutive activation of the apoptosome and caspase-3 (Figure 2). Next to secondary necrosis following apoptosis, cells can die directly by primary necrosis via different pathways (Table 1). For example, GSDMD is responsible for the execution of pyroptosis, an inflammasome-dependent necrotic cell death modality involving processing of pro-interleukin-1\(\beta\) (pro-IL-1\(\beta\)) by caspase-1\textsuperscript{56,66}. Pyroptosis is characterized by ballooning of the cell and release of processed IL-1\(\beta\). Similarly, GSDME executes primary necrosis as well. Chemotherapy treatment of different cancer cell lines results in caspase-3-mediated cleavage of GSDME and pyroptotic ballooning without passing through an apoptotic morphology\textsuperscript{25,33}, suggesting that necrotic plasma membrane permeabilization by GSDME in this case precedes the apoptotic process which seems paradoxical since the same upstream apoptotic machinery is triggered in case of secondary necrosis. This is probably due to different buffering capacities to restrain GSDME activation. Moreover, the simultaneous detection of biochemical markers for apoptosis and pyroptosis after chemotherapy treatment\textsuperscript{20} argues for concurrent occurrence of apoptosis and pyroptosis. In absence of GSDME, a prolonged apoptotic morphology and dominance of apoptotic markers is seen\textsuperscript{20,33}, indicating that GSDME activation induces the final membrane permeabilization, as an early event in case of pyroptosis and a late event in case of secondary necrosis. Whether this can be defined as real pyroptosis, is a matter of definition (Table 1). Pyroptosis s.s. is defined as inflammasome-dependent and associated with the release of IL-1\(\beta\) (previously called “pyrogen”) and more recently with caspase-1/4-mediated proteolytic activation of GSDMD\textsuperscript{66}. GSDME-mediated cell death mentioned above is not inflammasome-dependent and is therefore called “pyroptosis-like” (Table 1). All together these data suggest that caspase-3 mediated cleavage of GSDME results in necrotic cell death, either called secondary necrosis when following an apoptotic phase or called pyroptosis-like. However, the presence of cleaved GSDME is not always associated with cell death. Indeed, despite caspase-3-mediated cleavage and a clear apoptotic phenotype, N-GSDME apparently does not regulate secondary necrosis in human T-cells and monocytes\textsuperscript{67}. Interestingly, phosphorylation at threonine (Thr) 6 was recently reported to prevent GSDME pore formation even when processed, revealing an extra layer of regulation (Figure 2)\textsuperscript{65}.

**GSDME AND CANCER**

Currently no recurrent genetic mutations in GSDME were found in tumors\textsuperscript{21,23}. Instead, GSDME expression seems to be epigenetically regulated. GSDME promoter methylation, GSDME mRNA and protein expression were analyzed in different cancer types in different studies\textsuperscript{15,21–27,29–33,35–40,59}. For
detailed information on GSDME methylation/expression per tumor type for patient samples and cell lines see Table 2 and Supplementary Table 1, respectively.

**GSDME methylation is both a pan-cancer and cancer type specific biomarker**

The potential of GSDME methylation as a marker for cancer detection, was initially explored in two studies involving data from The Cancer Genome Atlas (TCGA) for breast and colorectal cancer\textsuperscript{21,28}. All of the 22 GSDME CpGs (Figure 3) interrogated by the Illumina 450K methylation array showed differential methylation between primary tumor and paired normal tissues. Increased methylation of promoter CpGs was observed in cancer compared to normal samples, concordant with GSDME’s suggested role as tumor suppressor gene. Furthermore, GSDME gene body methylation exhibited an opposite pattern to that in the promoter, namely a higher methylation in normal samples compared to cancer samples. Methylation levels of CpGs in the promoter region were highly correlated with each other, as was the case with CpGs in the gene body region, but not between these two distinct regions. Interestingly, methylation levels of a combination of two GSDME CpGs, one in the gene body and one in the promoter, performed exceptionally well as detection biomarker. In breast cancer, the final model reached a cross validated area under the curve (AUC) of 0.93, with a sensitivity of 85.3% without false positives and overall accuracy of 87\%\textsuperscript{21}. Moreover, colorectal adenocarcinomas are reliably predicted \textit{in silico} with a cross validated AUC of 0.95, sensitivity of 93.3\%, specificity of 93.7\% and overall accuracy of 97.6\% in the TCGA dataset\textsuperscript{28}. These predictions were unaffected by age and disease stage, making GSDME an excellent candidate for early detection irrespective of tumor stage.

The analysis of GSDME methylation as a cancer detection biomarker has been expanded using TCGA methylation datasets for 14 different types of cancer\textsuperscript{29}. A widespread hypermethylation of gene promoter CpGs and hypomethylation of gene body CpGs in different cancer tissues was reported. Combinations of six GSDME CpGs were able to predict cancer versus normal tissue accurately across different tumor types with an AUC of 0.87. Predictions in individual datasets using only three CpGs still resulted in AUCs ranging between 0.80 and 0.95, further highlighting GSDME’s potential as a pan-cancer detection biomarker. Interestingly, six of 22 CpGs were distinctly recurring in all high scoring CpG combinations. Moreover, around 75 000 combinations of six GSDME CpGs were tested for their ability to distinguish between different tissue types based on methylation in a combined dataset of more than 5000 tumor and 700 control tissues, exhibiting maximal AUC values ranging between 0.79 and 0.98 for predicting individual cancer types against all others, with esophageal cancer scoring the lowest and prostate, thyroid and colorectal cancer scoring the highest\textsuperscript{29}. Altogether, these data strongly suggest that GSDME methylation can be reliably used as both a pan-cancer and cancer type specific biomarker, highlighting the potential of GSDME methylation as a universal cancer detection biomarker.

**GSDME methylation has potential as prognostic biomarker in breast cancer**
Next to methylation differences between normal and cancer tissues, associations of GSDME methylation and different clinicopathological parameters were studied. In breast cancer, lobular adenocarcinomas have significantly higher GSDME promoter methylation values compared to ductal adenocarcinomas\textsuperscript{21}. In addition, a significant association of GSDME promoter methylation and tumor stage was observed, with stage III showing the highest methylation while stage I and II performed identical\textsuperscript{21}. Furthermore, a significant association with progesterone receptor (PR) and estrogen receptor (ER) status was found. GSDME promoter methylation was higher in PR+ breast adenocarcinomas compared to PR- ones, while for GSDME gene body methylation the opposite was true. A similar pattern as with PR was found for ER status\textsuperscript{21}. This association was not found in previous studies in patient samples\textsuperscript{21,36}, analyzing different CpGs in less samples with a different technique (Table 2). Instead, they reported a positive association with lymph node metastasis\textsuperscript{36} and with HER2 amplification\textsuperscript{22}. Surprisingly, GSDME gene body (not promoter) methylation, showed a (negative) association with 5-year overall survival time in ductal breast adenocarcinomas\textsuperscript{21}, revealing GSDME methylation as potential prognostic biomarker in breast cancer. In colorectal cancer this association was not found\textsuperscript{28}. Instead, a significant increase of GSDME promoter methylation was reported in tumors with lymphatic vessel invasion and high tumor-node-metastasis (TNM) stage\textsuperscript{39} as well as differential methylation between left sided and right sided colorectal cancer, with a higher methylation observed in right sided tissue\textsuperscript{28}. For gastric cancer, correlations of GSDME methylation with positivity for Epstein Barr virus, absence of metastasis and presence of the CpG island methylator phenotype (CIMP) were found\textsuperscript{35}. For most of these associations, the clinical importance is still unknown, but they may provide valuable information for further studies. Especially the association of GSDME gene body methylation with 5-year overall survival might have impact on clinical practice.

**No clear difference in GSDME expression between normal and cancer cells**

In addition to methylation, GSDME mRNA or protein expression were compared between cancer and normal tissue\textsuperscript{20,21,23,26,28,36,37} (Table 2). Mostly, GSDME expression is downregulated in cancer compared to normal samples\textsuperscript{21,23,36,37}. However, some studies reported no differences in GSDME mRNA\textsuperscript{28} nor protein\textsuperscript{20} expression between cancer and paired normal samples. In one study, GSDME protein expression was even higher in cancer compared to normal samples\textsuperscript{20}. These divergent results prevent uniform conclusions about the difference in GSDME expression between normal and cancer samples. Therefore, in contrast to methylation, GSDME expression does not provide a solid basis for a universal cancer detection marker\textsuperscript{21,28}. Despite the clear differences in GSDME methylation between cancer and normal tissue, no clear correlation between GSDME methylation and GSDME expression was found in patients\textsuperscript{21,23,28,36}.

**GSDME expression may have potential as prognostic marker in esophageal cancer**

For GSDME mRNA and protein expression, several associations with clinicopathological parameters were reported. Several studies found an inverse correlation between ER status and
GSDME expression\textsuperscript{15,21,36} (Table 2), forming the basis for another name for GSDME, namely ICERE (inversely correlated with estrogen receptor expression)\textsuperscript{15}. Moreover, a significantly higher GSDME expression in lobular adenocarcinomas as compared to ductal adenocarcinomas was reported in breast cancer\textsuperscript{21}. In lung adenocarcinoma, associations with EGFR, STK11 and KEAP1/NFEL2 mutation status were found\textsuperscript{20}. GSDME mRNA was modestly upregulated in EGFR-mutant neoplasms, but downregulated in STK11- or KEAP1/NFEL2-mutant tumors, as compared with the respective wild-type counterparts. In squamous esophageal cancer, GSDME protein expression level was positively correlated with a better prognosis\textsuperscript{26}. The 5-year survival rate of the GSDME high expression group was significantly higher compared to the GSDME low expression group, suggesting GSDME’s potential as prognostic biomarker in squamous esophageal cancer. The better outcome is explained by the potential of GSDME expressing esophageal cancer cells to die by pyroptosis following cisplatin and BI2536 co-treatment, which is a strongly immunogenic type of cell death\textsuperscript{26}. Remarkably, also other GSDME expressing cancer types such as melanoma have been reported to generate strong immune infiltration (see later)\textsuperscript{68}.

\textbf{Introduction of GSDME in cancer cell lines decreases cell growth}

In contrast to patient samples, a clear correlation between GSDME methylation and expression was found in several individual cell line experiments. After treatment with the demethylating agent 5-aza-2’-deoxycytidine or decitabine, cell lines that first did not express methylated GSDME alleles, now expressed GSDME after demethylation\textsuperscript{33,35–38}. Moreover, introduction of GSDME in cancer cell lines markedly decreased cell growth and colony forming ability\textsuperscript{24,35–37}. In contrast, knock down of GSDME increased cellular invasiveness and growth in vitro\textsuperscript{36,37,65}. Furthermore, different studies suggested the involvement of GSDME in p53-dependent pathways\textsuperscript{34,35,37,38}. GSDME is a target of the p53 family and especially p63γ\textsuperscript{38} as its expression can be upregulated by p63γ through direct interaction with the p53 response element of GSDME\textsuperscript{34}.

\textbf{The role of GSDME expression on tumor growth in mice is still ambiguous}

Despite promising in vitro studies, in vivo experiments in mice are inconclusive about the role of GSDME in tumor biology. Two independent intestinal cancer mouse models exhibited no major differences in tumor development between GSDME KO and WT mice, neither for the number of affected mice, nor for the multiplicity of proliferative lesions per mouse\textsuperscript{69}. Similarly, the size and weight of GSDME depleted xenograft tumors were comparable to WT xenograft tumors in colorectal cancer\textsuperscript{20}, lung cancer\textsuperscript{20} and melanoma\textsuperscript{30} models. However, in another melanoma study GSDME KO tumors formed and grew significantly faster than those expressing GSDME. This tumor suppressive activity of GSDME might be related to its ability to execute necrosis and potentiate caspase-3 activation through the release of cyt c from the mitochondria\textsuperscript{65} (Figure 2). In line with the enhanced and accelerated cell death in presence of GSDME, more severe inflammation was found in intestinal tumors in GSDME WT compared to GSDME KO mice\textsuperscript{69}. As GSDME expressing tumors also increase
macrophage-mediated phagocytosis and attract more tumor-infiltrating natural-killer and CD8+ T lymphocytes71, GSDME might be involved in creating a more inflammatory tumor microenvironment by induction of necrotic cell death69.

**GSDME as potential predictive biomarker in cancer: an important ally in chemotherapy treatment**

As GSDME protein expression does not always affect tumor volume and weight20,70, GSDME is probably not directly involved in tumor development. Nevertheless, its presence seems an important determinant for the type of cell death induced by chemotherapy, thereby influencing the efficiency of the chemotherapy treatment. Several cancer cell lines that do express GSDME show caspase-3-dependent GSDME activation following chemotherapy treatment20,25,27,30,32,33,70,72,73, e.g. SH-SY5Y (neuroblastoma) following doxorubicin33 or dasatinib72, etoposide treated MeWo (skin melanoma)30,33 cell lines and cisplatin + BIX-01294 treated SGC-7901 (shown to be a HeLa derivative (endocervical adenocarcinoma))73. These cells exhibit a necrotic morphology, characterized by swelling and direct lysis of the plasma membrane, thereby releasing their content into the tumor microenvironment (Table 1). Remarkably, in absence of GSDME expression (e.g. as is the case in Jurkat cells), the same treatment induces apoptosis characterized by cell shrinkage, plasma membrane blebbing and the release of apoptotic bodies (Table 1)13,70. In addition, GSDME expression affects chemotherapy efficiency as GSDME knockdown in A-549 cells attenuated cisplatin-induced cell death compared to WT cells32. Similarly, combined administration of sulfasalazine with iron dextran no longer inhibited A-375 xenograft tumor growth after GSDME knock down31. Furthermore, Ceritinib performs partially impaired treatment efficacy upon GSDME KO in NCI-H3122 cells20. An improvement of therapeutic index was observed as well in case of exogenous GSDME expression in HCC827 cells20. Moreover, combined treatment of decitabine, a DNA methyltransferase inhibitor elevating GSDME expression, with chemotherapy or phototherapy improved anti-tumor treatment efficiencies33,74,75. Nevertheless, in some cases GSDME expression had no effect on cell survival after chemotherapy treatment25,70, complicating the role of GSDME in chemotherapy-induced cell death. An interesting finding in that respect is that the pore-forming activity of GSDME is prevented by phosphorylation at Thr665. As GSDMA, a close relative of GSDME, is phosphorylated by Polo like kinase 1 (Plk1) at Thr876, the same kinase might inactivate GSDME65. Plk1 is a known oncogene that is often activated in cancer cells, suggesting a second way of inactivation of GSDME in cancer cells, next to methylation. Remarkably, co-treatment of the Plk1 inhibitor BI2536 with cisplatin sensitizes esophageal cancer cells, which show a high intrinsic GSDME expression and GSDME cleavage after cisplatin treatment26. This might indicate that despite high GSDME expression in these cells, GSDME cannot execute its cell death function due to phosphorylation by Plk1, and that inhibition of GSDME phosphorylation can intensify the response to chemotherapy treatment. Moreover, in those cases where GSDME depletion didn’t affect tumor formation in treated cancer cells, it did reduce the release of pro-inflammatory factors, including IL-1β and lactate dehydrogenase (LDH)25,70, changing the inflammatory status of the tumor microenvironment. Furthermore, GSDME depletion reduces tissue injury and inflammation in the
lungs, spleen and gastrointestinal tract after chemotherapy in healthy mice, again suggesting that
GSDME-mediated cell death influences the extent of inflammation. Moreover, implanted GSDME-
deficient melanoma tumors show impaired HMGB1 release and reduced tumor-associated T cell and
activated dendritic cell infiltrates in response to BRAFi + MEKi treatment compared to the control
counterparts. As GSDME KO tumors also showed more frequent tumor regrowth after BRAFi +
MEKi removal, GSDME dependent inflammation around the tumor can be considered anti-
tumorigenic.

Next to chemotherapeutics, the efficiency of other therapeutics is influenced by GSDME
eexpression. For example, treatment of CCRF-CEM cells with glucocorticoids induces GSDME
expression followed by cell death and enhancement of caspase-3 activation. As glucocorticoids are
used, in combination with other therapeutics, for the treatment of lymphoid malignancies, the
expression of GSDME in these malignancies might be an important factor in their response to this
kind of therapy. In conclusion, GSDME expression sometimes correlates with tumor growth, but often
contributes to therapeutic efficiency and is therefore an important ally in (chemotherapy) treatment.

CONCLUSION

Overall, GSDME shows a broad applicability in cancer diagnosis, monitoring and therapy. Especially
GSDME methylation shows strong potential as detection biomarker in different cancer types. The
methylation of different CpG combinations proved diagnostically useful in predicting cancer versus
normal tissue accurately across 14 different tumor types, irrespective of tissue type, highlighting the
potential of GSDME methylation as a pan-cancer biomarker. Furthermore, other combinations were
able to differentiate between different types of cancer. Therefore, GSDME methylation patterns and
their generalizability over different tumor types could form the basis of a minimally invasive
biomarker assay for early cancer detection. In addition to detection, GSDME methylation and protein
expression may show promise as prognostic markers. To evaluate this, current studies should be
expanded to more tumor types, as until now the potential of GSDME as prognostic marker is only
investigated in breast, colorectal and esophageal cancer. Moreover, large prospective studies, with
homogenous cancer populations are needed.

A next step to develop GSDME methylation as a minimally invasive pan-cancer biomarker could be
the analysis of GSDME methylation in liquid biopsies. A liquid biopsy is defined as the analysis of
tumor material (e.g. cells or nucleic acids) obtained through sampling of blood or other body fluids.
One approach to identify tumor specific (epi)genetic aberrations is the analysis of tumor DNA present
in plasma, called circulating tumor DNA (ctDNA). ctDNA is released primarily via dying tumor cells,
which may occur throughout a tumor, giving a more representative picture of the tumor genome
compared to single biopsies. Moreover, it is believed that ctDNA is readily detected in plasma of even
early stage cancer patients. Several studies have provided proof of principle for the detection of tumor specific methylation changes on ctDNA.\textsuperscript{77-79}

For the association of GSDME mRNA and protein expression with cancer, the conclusions are less clear, hampering the use of GSDME expression as detection marker. Given a higher promoter methylation, most studies report a downregulation of GSDME expression in cancer as compared to normal tissues. Nevertheless, identical or even higher GSDME expression in cancer compared to normal samples has been found as well, which seems contrary to GSDME’s potential tumor suppressive function. However, recent insights argue for a second possibility, viz. that despite its processing by caspase-3, mechanisms exist that keep GSDME inactive. While methylation of specific CpGs in the GSDME gene inhibits its expression, post-translational phosphorylation of Thr6 in the GSDME protein prevents its pore forming capacity in plasma membranes and mitochondria. The kinases responsible for direct phosphorylation of this residue have not yet been characterized, but PLK1 seems to be a likely candidate, as it induces phosphorylation of many cellular proteins including GSDMA. Importantly, PLK1 is an oncogene, which counteracts the potential tumor suppressor activity of GSDME.

Finally, recent breakthroughs on the function of the GSDMs have shed new light on the importance of GSDME expression in cancer and cancer treatment. Growing evidence suggests that GSDME indirectly acts as a tumor suppressor by promoting a more inflammatory and immunogenic microenvironment via the release of cellular content such as danger- or damage-associated molecular patterns (DAMPs), chemokines and cytokines. Nevertheless, pending on the stage of tumorigenesis, inflammation can have pro- or anti-tumorigenic effects. On the one hand, inflammation attracts e.g. natural killer and CD8+ T cells to the tumor site, which are able to eliminate cancer cells. On the other hand, tumor-associated macrophages and regulatory T cells can be attracted and dampen the effect of innate and adaptive effector immune cells at various levels through different mechanisms. As GSDME expressing tumors are shown to attract more natural killer and CD8+ cells, which act anti-tumorigenic, it makes sense that cancer cells are selected that silence GSDME, resulting in a more hidden niche for the immune system. Moreover, several studies pointed GSDME expression levels as an important determinant in response to chemotherapy, thereby influencing therapeutic efficacy. Therefore, the potential of GSDME as new therapeutic target to boost the immunogenicity of cancer death should be studied in more detail. For instance, therapeutic induction and activation of GSDME can be of clinical value to turn ‘cold’ tumors, which contain few infiltrating T cells, into ‘hot’ ones, containing high levels of infiltrating T cells and more antigen processing, all contributing to an improved response to immunotherapy. As proof of concept, the controlled release of N-GSDMA3 from an antibody-drug conjugate selectively into tumor cells in mice using a bioorthogonal chemical system, was shown to enhance anti-tumor responses such as increased CD3+ T cell infiltration.\textsuperscript{80} Moreover, induction of pyroptosis in only 15% of the cells proved sufficient to clear the entire tumor graft,\textsuperscript{80} emphasizing the need for selective delivery methods, specific small-molecule GSDME activators or gene therapy methods for direct induction of pyroptotic cell death. However, caution is advised as GSDME
mediated pyroptosis is not always beneficial. Activation of GSDME mediated pyroptosis by chimeric antigen receptor (CAR) T cells was recently shown to activate caspase-1 and subsequent GSDMD in macrophages during CAR T cell therapy, leading to extensive cytokine release and cytokine release syndrome eliciting undesirable side effects in patients\textsuperscript{81}. Altogether, more fundamental research on the biology of GSDME is required to unravel its full clinical potential.

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**Conflict of Interest**

The authors declare no conflict of interest.
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A PLK1 kinase inhibitor enhances the chemosensitivity of cisplatin by inducing pyroptosis in oesophageal squamous cell carcinoma. *EBioMedicine* 2019;41:244–55.


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**LEGENDS OF FIGURES**

Figure 1. Original hypothesis of the function of GSDME in hearing loss and cancer.

Figure 2. Cell death pathways wherein GSDME is involved.
Apoptosis can be triggered by internal or external stimuli, leading to the activation of the intrinsic or extrinsic apoptotic pathway respectively. In the intrinsic apoptotic pathway, mitochondrial outer membrane permeabilization (MOMP) by Bak/Bax is triggered by internal stress such as DNA damage or oxidative stress, causing the release of cyt c into the cytoplasm. Cyt c initiates the formation of the apoptosome which facilitates the autocleavage of caspase-9, which activates caspase-3, a common executioner caspase, involved in both the intrinsic and extrinsic apoptotic pathway. In the extrinsic apoptotic pathway, activation of death receptors by ligand binding results in the consecutive activation of caspase-8 and caspase-3. Caspase-3 cleaves GSDME in its hinge-region, thereby releasing the N-terminus from the inhibitory C-terminal domain. Next, N-GSDME targets both the mitochondrial and the plasma membrane. By targeting the mitochondrial membrane, more cyt c is released from the mitochondria, resulting in a self-amplification loop by activation of caspase-3 and GSDME. On the other hand, the increasing plasma membrane permeabilization results in necrotic cell death. If GSDME becomes phosphorylated, N-GSDME oligomerization and pore formation are prevented.

**Figure 3. The GSDME gene with annotation of the CpGs analyzed in the different studies.**

The GSDME gene layout shows the CpGs interrogated by the different studies. The upper track outlines the full scope of the GSDME gene which extends from 24 737 972 to 24 809 244 on chromosome 7. The lower track is a zoomed-in section of the promoter region where several of the interrogated CpGs are located. Translation and transcription start sites are indicated by the red and yellow pins respectively. The 22 CpGs analyzed in the TCGA study are depicted in dark green, while CpGs 1-4 were analyzed by pyrosequencing in the study of Croes et al. CpGs 5-8 were the ones studied by Fujikane et al. The pink qtaqman probe was used in the studies of Kim et al. The brown bar delimits the 514 bp region where Akino et al interrogated CpGs. All annotations are based on the “Regulatory build of the GSDME gene” in Ensembl, using the Human Genome Feb. 2009 (GRCh37/hg19) assembly.
Table 1. Overview of the different characteristics and corresponding morphology of GSDM-mediated cell death modalities.

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Primary Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shrinking of cytoplasm and condensation of nucleus</td>
<td>Cell swelling, permeabilization and rupture of the plasma membrane</td>
</tr>
<tr>
<td>Formation of apoptotic bodies (membrane contained vesicles enclosing elements of cytosol, organelles and nuclear material)</td>
<td>Differential leakage of cellular content</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Secondary Necrosis</th>
<th>Pyroptosis - like</th>
<th>Pyroptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>No loss of membrane integrity; apoptotic caspases are crucial for the apoptotic containment program</td>
<td>Associated with cell swelling (oncasis) and plasma membrane permeabilization of cells that started the apoptotic program</td>
<td>Formation of large pyroptotic bodies</td>
<td>Formation of large pyroptotic bodies</td>
</tr>
<tr>
<td>Efficient phagocytosis of apoptotic cells and fragments</td>
<td>Occurs in case of inefficient clearance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptotic caspases dependent</td>
<td>Proteolytic activation of GSDME by caspase-3</td>
<td>Direct activation of GSDME by caspase-3 without apoptotic phase</td>
<td>Inflammasome dependent, inflammatory caspase-1/4 are crucial for proteolytic activation of GSDMD</td>
</tr>
<tr>
<td></td>
<td>Release of DAMPs, chemokines and cytokines</td>
<td>Release of DAMPs, chemokines and cytokines</td>
<td>Proteolytic activation of pro-IL-1β. Release of DAMPs, chemokines and cytokines</td>
</tr>
<tr>
<td>Less immunogenic</td>
<td>Immunogenic?</td>
<td>Immunogenic</td>
<td>Immunogenic</td>
</tr>
</tbody>
</table>

DAMPs: Danger-associated molecular patterns
Table 2. *GSDME* and cancer – patient studies

<table>
<thead>
<tr>
<th>Reference</th>
<th>Analysis</th>
<th>Technique</th>
<th>Number of samples</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BREAST CANCER</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Croes et al, 2018$^{21}$</td>
<td>Methylation</td>
<td>TCGA - Infinium HumanMethylation450k array (22 CpGs in <em>GSDME</em>)</td>
<td>668 cancer samples 85 paired normal breast samples</td>
<td>Higher <em>GSDME</em> promoter methylation (14/14 CpGs) in cancer compared to normal samples ($p$ value range: $9.8 \times 10^{-14} - 2.2 \times 10^{-4}$) Lower <em>GSDME</em> gene body methylation (6/6 CpGs) in cancer compared to normal samples ($p$ value range: $1 \times 10^{-12} - 4.5 \times 10^{-3}$)</td>
</tr>
<tr>
<td></td>
<td>Gene expression</td>
<td>TCGA - Agilent 244K Custom Gene Expression array</td>
<td>476 cancer samples 56 normal breast samples</td>
<td>Lower <em>GSDME</em> expression in cancer compared to normal samples ($p$ value: $1.8 \times 10^{-9}$ (array); $p$ value: $2.2 \times 10^{-16}$ (RNA-seq))</td>
</tr>
<tr>
<td></td>
<td>RNA-sequencing</td>
<td></td>
<td>666 cancer samples 71 normal breast samples</td>
<td>Mean <em>GSDME</em> expression: cancer samples: -1.8 (array); 7.2 (RNA-seq) normal samples: -0.99 (array), 8.2 (RNA-seq)</td>
</tr>
<tr>
<td>Stoll et al, 2017$^{23}$</td>
<td>Methylation</td>
<td>TCGA – not specified (16 CpGs in <em>GSDME</em> promoter)</td>
<td>743 breast cancer samples 98 normal breast samples</td>
<td><em>GSDME</em> promoter hypermethylation not explaining <em>GSDME</em> expression</td>
</tr>
<tr>
<td></td>
<td>Gene expression</td>
<td>TCGA – not specified</td>
<td>not specified</td>
<td>Lower <em>GSDME</em> expression in cancer compared to normal samples ($p$ value: $2.1 \times 10^{-7}$) Lower <em>GSDME</em> expression for all groups of breast cancers (ER+, HER2+/ER-, triple negative) ($p$ value range: $2.4 \times 10^{-7} - 9.3 \times 10^{-9}$)</td>
</tr>
<tr>
<td></td>
<td>METABRIC – not specified</td>
<td>not specified</td>
<td></td>
<td>Lower <em>GSDME</em> expression in cancer compared to normal samples ($p$ value: $1.1 \times 10^{-12}$) Lower <em>GSDME</em> expression for all groups of breast cancers (luminal A, luminal B, HER2, basal) ($p$ value range: $1.1 \times 10^{-22} - 0.0083$)</td>
</tr>
<tr>
<td>Reference</td>
<td>Analysis</td>
<td>Technique</td>
<td>Number of samples</td>
<td>Result</td>
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<tr>
<td>Croes <em>et al</em>, 2017</td>
<td>Methylation</td>
<td>Pyrosequencing</td>
<td>123 cancer samples</td>
<td>Higher GSDME promoter methylation in cancer compared to normal samples (<em>p value</em>: 6.1*10^{-4}) Median GSDME methylation: cancer samples: 12% [range: 0%–96%] normal samples: 4% [range: 1%–7%] No significant differences between paired cancer and normal breast tissues median GSDME methylation difference: 3.5% [range: -29%–73%]</td>
</tr>
<tr>
<td>Fujikane <em>et al</em>, 2010</td>
<td>Methylation</td>
<td>Pyrosequencing</td>
<td>73 cancer samples</td>
<td>Higher GSDME promoter methylation in cancer compared to normal samples (<em>p &lt; 0.001</em>) Mean GSDME methylation: cancer samples: 8.5; 95% CI [6.2–10.8] normal samples: 3.4; 95% CI [2.5–4.3] No significant differences between the paired cancer and normal breast tissues Mean GSDME methylation: cancer samples: 7.3; 95% CI [2.3–12.3] normal samples: 3.5; 95% CI [2.5–4.5]</td>
</tr>
<tr>
<td>Kim <em>et al</em>, 2008</td>
<td>Methylation</td>
<td>TaqMan-MSP</td>
<td>34 cancer samples</td>
<td>GSDME promoter: more often methylated in cancer compared to normal samples (<em>p value</em>: 0.006) GSDME methylated in: 18/34 (53%) cancer samples 2/13 (15.3%) paired normal breast samples 0/7 (0%) healthy normal breast samples</td>
</tr>
<tr>
<td>Reference</td>
<td>Analysis</td>
<td>Technique</td>
<td>Number of samples</td>
<td>Result</td>
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<tr>
<td></td>
<td>Gene expression</td>
<td>Real-time RT-PCR</td>
<td>1 cancer sample</td>
<td>Lower GSDME expression in cancer compared to normal samples (paired: ( p \text{ value} = 0.003 ); unpaired: ( p \text{ value} = 0.002 ))</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 paired normal breast sample</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 normal breast sample*</td>
<td></td>
</tr>
<tr>
<td>Thompson and Weigel, 1998</td>
<td>Gene expression</td>
<td>Cancer Profiling Array</td>
<td>10 paired cancer – normal breast samples</td>
<td>Lower GSDME expression in 6/10 (60%) of cancer compared to normal samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Semi-quantitative RT-PCR</td>
<td>29 cancer samples: 15 ER+ 14 ER- 2 normal breast samples (ER-)</td>
<td>Lower GSDME expression in ER+ compared to ER- breast samples (( p &lt; 0.001 ))</td>
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<tr>
<td>COLORECTAL CANCER</td>
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</tr>
<tr>
<td>Ibrahim et al, 2019(^{28})</td>
<td>Methylation</td>
<td>TCGA - Infinium HumanMethylation450k array (22 CpGs in GSDME)</td>
<td>389 cancer samples 43 paired normal colon samples</td>
<td>Higher GSDME promoter (12/14 CpGs) methylation in cancer compared to normal samples (( p \text{ value range: } 1.7<em>10^{-6} - 0.025 )) Lower GSDME gene body methylation (5/6 CpGs) in cancer compared to normal samples (( p \text{ value range: } 8.3</em>10^{-9} - 4.5*10^{-3} ))</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCGA - Agilent 244K Custom Gene Expression array</td>
<td>221 cancer samples 20 normal colon samples</td>
<td>No significant differences in GSDME expression between cancer samples and normal samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RNA-sequencing</td>
<td>437 cancer samples 39 normal colon samples</td>
<td>Mean GSDME expression: cancer samples: (-0.46) (array); 5.45 (RNA-seq) normal samples: (-3.18) (array), 5.8 (RNA-seq)</td>
</tr>
<tr>
<td>Yokomizo et al, 2012(^{29})</td>
<td>Methylation</td>
<td>qMSP</td>
<td>85 cancer samples 85 paired normal colorectal samples</td>
<td>GSDME methylated in: 29/85 (34%) cancer samples ( \text{No results for normal samples} )</td>
</tr>
<tr>
<td>Kim et al, 2008(^{37})</td>
<td>Methylation</td>
<td>COBRA</td>
<td>10 cancer samples 9 paired normal colorectal samples</td>
<td>GSDME methylated in: 4/10 (40%) cancer samples ( 0/9 ) paired normal colorectal samples</td>
</tr>
<tr>
<td>Reference</td>
<td>Analysis</td>
<td>Technique</td>
<td>Number of samples</td>
<td>Result</td>
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<tr>
<td></td>
<td></td>
<td>Bisulfite sequencing</td>
<td>5 cancer samples</td>
<td>GSDME methylated in: 5/5 (100%) cancer samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 paired normal colorectal samples</td>
<td>0/10 (0%) paired normal colorectal samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TaqMan-MSP (cut-off: 0.65)</td>
<td>100 cancer samples</td>
<td>GSDME promoter: more often methylated in cancer compared to normal samples (p &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100 paired normal colorectal samples</td>
<td>GSDME methylated in: 65/100 (65%) cancer samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11 normal colorectal samples*</td>
<td>3/100 (3%) paired normal colorectal samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/11 (9%) normal colorectal samples*</td>
</tr>
<tr>
<td></td>
<td>Gene expression</td>
<td>Real Time RT-PCR</td>
<td>5 cancer sample</td>
<td>4/5 cancer samples reduced GSDME expression compared to paired normal samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 paired normal breast sample</td>
<td>GSDME expression in cancer 5x lower than in normal colon sample* (p value: 0.007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 normal colon sample*</td>
<td></td>
</tr>
</tbody>
</table>

**GASTRIC CANCER**

| Akino et al, 2006<sup>[5]</sup> | Methylation       | COBRA                      | 89 cancer samples                   | 46/89 (52%) cancer samples: increased GSDME methylation               |
|                                 |                   |                            | 89 paired normal gastric samples   | 0/89 paired normal gastric samples: GSDME barely detectable (~0%)        |
|                                 |                   |                            |                                    | 46 samples shown to be methylated by COBRA: all analyzed CpG sites densely methylated |
|                                 | Gene expression   | qRT-PCR                    | 10 cancer samples                  | Methylation GSDME: Almost no GSDME expression (N = 5)                   |
|                                 |                   |                            |                                    | Unmethylated GSDME: varying levels of GSDME expression (N = 5)         |
| Kim et al, 2008<sup>[5]</sup>  | Methylation       | TaqMan-MSP (cut-off: 1)    | 31 cancer samples                  | GSDME methylated in: 17/31 (54%) cancer samples                       |
|                                 |                   |                            | 11 paired normal gastric samples   | 1/11 (9%) paired normal gastric samples                               |

**ESOPHAGEAL CANCER**
<table>
<thead>
<tr>
<th>Reference</th>
<th>Analysis</th>
<th>Technique</th>
<th>Number of samples</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wu et al, 2019&lt;sup&gt;26&lt;/sup&gt;</td>
<td>Protein expression</td>
<td>IHC (tissue microarray)</td>
<td>105 cancer samples</td>
<td>Higher GSDME expression in cancer compared to normal esophageal samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>75 normal esophageal samples</td>
<td></td>
</tr>
<tr>
<td>Kim et al, 2008&lt;sup&gt;36&lt;/sup&gt; (Supplementary Material)</td>
<td>Methylation</td>
<td>TaqMan-MSP (cut-off: 0.001)</td>
<td>18 cancer samples</td>
<td>GSDME methylated in:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 paired normal esophageal samples</td>
<td>2/18 (11.1%) cancer samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0/20 (0%) paired normal esophageal samples</td>
</tr>
<tr>
<td><strong>BLADDER CANCER</strong></td>
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</tr>
<tr>
<td>Kim et al, 2008&lt;sup&gt;36&lt;/sup&gt;</td>
<td>Methylation</td>
<td>TaqMan-MSP (cut-off: 0.001)</td>
<td>55 cancer samples</td>
<td>GSDME methylated in:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 paired normal bladder samples</td>
<td>12/55 (21.8%) cancer samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0/30 (0%) paired normal bladder samples</td>
</tr>
<tr>
<td><strong>LUNG CANCER</strong></td>
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<tr>
<td>Lu et al, 2018&lt;sup&gt;30&lt;/sup&gt;</td>
<td>Protein expression</td>
<td>Western blot (10 EGFR+ and 10 EGFR-)</td>
<td>20 cancer samples</td>
<td>Ubiquitous GSDME expression in all samples, both normal and cancer samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 paired normal samples</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TMA</td>
<td>208 lung cancer samples of varying histotypes</td>
<td>GSDME pervasive expressed in 58.9% of TMA cases</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IHC</td>
<td>155 lung cancer samples:</td>
<td>GSDME pervasive expressed in:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 KRAS-mutant</td>
<td>60.0% KRAS-mutant cases</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>103 EGFR-mutant</td>
<td>67.0% EGFR-mutant cases</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>37 ALK-rearranged</td>
<td>56.8% ALK-mutant cases</td>
</tr>
</tbody>
</table>

*: Normal samples from people without cancer; TCGA: The cancer genome atlas; METABRIC: Molecular taxonomy of breast cancer international consortium; COBRA: Combined bisulfite restriction analysis; MSP: Methylation specific PCR; TMA: Tissue microarray, IHC: Immunohistochemistry
GSDME

Activation by genetic mutations → Increased regulated cell death → Hearing loss

Inactivation by promoter methylation → Decreased regulated cell death → Cancer

Decreased regulated cell death → Cancer