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

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;

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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[1]. 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[2–12]. *GSDME* belongs to the gasdermin (*GSDM*) family, which owes its nomenclature to its high expression pattern along the gastrointestinal tract and skin (dermis)[13,14]. In addition, expression of *GSDME* is reported in all vital organs[1,15]. Until now, six *GSDM* genes have been identified in humans: *GSDMA*, *GSDMB*, *GSDMC*, *GSDMD*, *GSDME* and *Pejvakin* (*PJVK*)[14]. Except for PJVK, all GSDM proteins consist of a conserved N- and C-terminal globular domain, separated by a flexible hinge region[16]. Recently, the N-terminal (N-GSDM) domain of GSDMA, -D and -E was shown to execute cell death by pore formation[17], 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[3,4,16,18,19] (Figure 1).

Next to hearing loss, *GSDME* has been associated with cancer[15,16,20–40]. Genomic methylation screens unveiled *GSDME* as a possible tumor suppressor gene[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[41,42]. In that respect it was hypothesized that DNA promoter methylation of *GSDME* prevents GSDME-mediated regulated cell death and in that way contributes to tumorigenesis (Figure 1). However, recent breakthroughs on the function of the *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 *GSDME* methylation/expression as a detection, prognostic and predictive cancer biomarker. Finally, the effect of *GSDME* protein expression on chemotherapeutic treatment will be explored. Overall we conclude that *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.

FUNCTION OF GSDME

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[43,44], asthma[45–48], hearing loss[1,49] and cancer[13,50–55]. The conserved N-terminal domain of all GSDMs but PJVK is shown to execute necrotic cell death[17]. Under physiologically normal conditions, this cytotoxic function is impeded by C-GSDM[16,56]. Depending on the cell death trigger, GSDMs are activated by proteolytic cleavage by different proteases, thereby liberating N-GSDM[33,46,56–62]. GSDME is cleaved by the crucial apoptotic executioner caspase-3[33,59] (Figure 2). In essence, apoptosis is a containment program preparing the dead cell corpse to be removed by phagocytosis[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)[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)[59]. When the *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)[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-1β (pro-IL-1β) by caspase-1[56,66]. Pyroptosis is characterized by ballooning of the cell and release of processed IL-1β. 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[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[20] argues for concurrent occurrence of apoptosis and pyroptosis. In absence of GSDME, a prolonged apoptotic morphology and dominance of apoptotic markers is seen[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-1β (previously called "pyrogen") and more recently with caspase-1/4-mediated proteolytic activation of GSDMD[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[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)[65].

GSDME AND CANCER

Currently no recurrent genetic mutations in *GSDME* were found in tumors[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[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[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%[21]. Moreover, colorectal adenocarcinomas are reliably predicted *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[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[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[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[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[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[21]. This association was not found in previous studies in patient samples[22,36], analyzing different CpGs in less samples with a different technique (Table 2). Instead, they reported a positive association with lymph node metastasis[36] and with *HER2* amplification[22]. Surprisingly, *GSDME* gene body (not promotor) methylation, showed a (negative) association with 5-year overall survival time in ductal breast adenocarcinomas[21], revealing *GSDME* methylation as potential prognostic biomarker in breast cancer. In colorectal cancer this association was not found[28]. Instead, a significant increase of *GSDME* promoter methylation was reported in tumors with lymphatic vessel invasion and high tumor-node-metastasis (TNM) stage[39] as well as differential methylation between left sided and right sided colorectal cancer, with a higher methylation observed in right sided tissue[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[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[20,21,23,26,28,36,37] (Table 2). Mostly, *GSDME* expression is downregulated in cancer compared to normal samples[21,23,36,37]. However, some studies reported no differences in *GSDME* mRNA[28] nor protein[20] expression between cancer and paired normal samples. In one study, *GSDME* protein expression was even higher in cancer compared to normal samples[26]. 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[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[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[15,21,36] (Table 2), forming the basis for another name for *GSDME*, namely *ICERE* (*inversely correlated with estrogen receptor expression*)[15]. Moreover, a significantly higher *GSDME* expression in lobular adenocarcinomas as compared to ductal adenocarcinomas was reported in breast cancer[21]. In lung adenocarcinoma, associations with *EGFR*, *STK11* and *KEAP1*/*NFEL2* mutation status were found[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[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[26]. Remarkably, also other *GSDME* expressing cancer types such as melanoma have been reported to generate strong immune infiltration (see later)[68].

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[33,35–38]. Moreover, introduction of GSDME in cancer cell lines markedly decreased cell growth and colony forming ability[24,35–37]. In contrast, knock down of *GSDME* increased cellular invasiveness and growth *in vitro*[36,37,65]. Furthermore, different studies suggested the involvement of GSDME in p53-dependent pathways[34,35,37,38]. *GSDME* is a target of the p53 family and especially p63γ[38] as its expression can be upregulated by p63γ through direct interaction with the p53 response element of *GSDME*[34].

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[69]. Similarly, the size and weight of *GSDME* depleted xenograft tumors were comparable to WT xenograft tumors in colorectal cancer[70], lung cancer[20] and melanoma[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[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[69]. As GSDME expressing tumors also increase

macrophage-mediated phagocytosis and attract more tumor-infiltrating natural-killer and CD8+ T lymphocytes[71], GSDME might be involved in creating a more inflammatory tumor microenvironment by induction of necrotic cell death[69].

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

As GSDME protein expression does not always affect tumor volume and weight[20,70], 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 treatment[20,25,27,30,32,33,70,72,73], e.g. SH-SY5Y (neuroblastoma) following doxorubicin[33] or dasatinib[72], 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 apoptotis characterized by cell shrinkage, plasma membrane blebbing and the release of apoptotic bodies (Table 1)[33,70]. In addition, GSDME expression affects chemotherapy efficiency as *GSDME* knockdown in A-549 cells attenuated cisplatin-induced cell death compared to WT cells[32]. Similarly, combined administration of sulfasalazine with iron dextran no longer inhibited A-375 xenograft tumor growth after *GSDME* knock down[31]. Furthermore, Ceritinib performs partially impaired treatment efficacy upon *GSDME* KO in NCI-H3122 cells[20]. An improvement of therapeutic index was observed as well in case of exogenous *GSDME* expression in HCC827 cells[20]. Moreover, combined treatment of decitabine, a DNA methyltransferase inhibitor elevating *GSDME* expression, with chemotherapy or phototherapy improved anti-tumor treatment efficiencies[33,74,75]. Nevertheless, in some cases *GSDME* expression had no effect on cell survival after chemotherapy treatment[25,70], 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 Thr6[65]. As GSDMA, a close relative of GSDME, is phosphorylated by Polo like kinase 1 (Plk1) at Thr8[76], the same kinase might inactivate *GSDME*[65]. 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 treatment[26]. 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[33], again suggesting that *GSDME*-meditated 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[68]. 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* expression. For example, treatment of CCRF-CEM cells with glucocorticoids induces *GSDME* expression followed by cell death and enhancement of caspase-3 activation[40,65]. 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[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[80]. Moreover, induction of pyroptosis in only 15% of the cells proved sufficient to clear the entire tumor graft[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[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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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[21,23,28], while CpGs 1-4 were analyzed by pyrosequencing in the study of Croes et *al*[22]. CpGs 5-8 were the ones studied by Fujikane *et al*[38]. The pink taqman probe was used in the studies of Kim *et al*[36,37]. The brown bar delimits the 514 bp region where Akino *et al* interrogated CpGs[35]. 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.

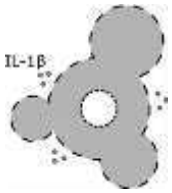
Apoptosis		**Primary Necrosis**	
Shrinking of cytoplasm and condensation of nucleus		Cell swelling, permeabilization and rupture of the plasma membrane	
Formation of apoptotic bodies (membrane contained vesicles enclosing elements of cytosol, organelles and nuclear material)		Differential leakage of cellular content	
Apoptosis	**Secondary Necrosis**	**Pyroptosis - like**	**Pyroptosis**
No loss of membrane integrity; apoptotic caspases are crucial for the apoptotic containment program	Associated with cell swelling (oncosis) and plasma membrane permeabilization of cells that started the apoptotic program	Formation of large pyroptotic bodies	Formation of large pyroptotic bodies
Efficient phagocytosis of apoptotic cells and fragments	Occurs in case of inefficient clearance		
Apoptotic caspases dependent	Proteolytic activation of GSDME by caspase-3	Direct activation of GSDME by caspase-3 without apoptotic phase	Inflammasome dependent, inflammatory caspase-1/4 are crucial for proteolytic activation of *GSDMD*
	Release of DAMPs, chemokines and cytokines	Release of DAMPs, chemokines and cytokines	Proteolytic activation of pro-IL-1β. Release of DAMPs, chemokines and cytokines
Less immunogenic	Immunogenic?	Immunogenic	Immunogenic
			

Table 2. *GSDME* and cancer – patient studies

Reference	Analysis	Technique	Number of samples	Result
BREAST CANCER				
Croes *et al*, 2018[21]	Methylation	TCGA - Infinium HumanMethylation450k array (22 CpGs in *GSDME*)	668 cancer samples 85 paired normal breast samples	Higher *GSDME* promoter methylation (14/14 CpGs) in cancer compared to normal samples (*p value* range: $9.8*10^{-14}$ – $2.2*10^{-4}$) Lower *GSDME* gene body methylation (6/6 CpGs) in cancer compared to normal samples (*p value* range: $1*10^{-12}$ – $4.5*10^{-3}$)
	Gene expression	TCGA - Agilent 244K Custom Gene Expression array	476 cancer samples 56 normal breast samples	Lower *GSDME* expression in cancer compared to normal samples (*p value*: $1.8*10^{-9}$ (array); *p value*: $2.2*10^{-16}$ (RNA-seq))
		RNA-sequencing	666 cancer samples 71 normal breast samples	Mean *GSDME* expression: cancer samples: -1.8 (array); 7.2 (RNA-seq) normal samples: -0.99 (array), 8.2 (RNA-seq)
Stoll *et al*, 2017[23]	Methylation	TCGA – *not specified* (16 CpGs in *GSDME* promoter)	743 breast cancer samples 98 normal breast samples	*GSDME* promoter hypermethylation not explaining *GSDME* expression
	Gene expression	TCGA – not specified	*not specified*	Lower *GSDME* expression in cancer compared to normal samples (*p value*: $2.1*10^{-9}$) Lower *GSDME* expression for all groups of breast cancers (ER+, HER2+/ER-, triple negative) (*p value* range: $2.4*10^{-7}$ – $9.3*10^{-5}$)
		METABRIC – not specified	*not specified*	Lower *GSDME* expression in cancer compared to normal samples (*p value:* $1.1*10^{-12}$) Lower *GSDME* expression for all groups of breast cancers (luminal A, luminal B, HER2, basal) (*p value* range: $1.1*10^{-22}$ – 0.0083)

Reference	Analysis	Technique	Number of samples	Result
Croes *et al*, 2017[22]	Methylation	Pyrosequencing (cut-off for positive methylation: 7%)	123 cancer samples 24 normal breast samples* 16 paired normal breast samples	Higher *GSDME* promoter methylation in cancer compared to normal samples (*p value*: $6.1*10^{-4}$) 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%]
Fujikane *et al*, 2010[38]	Methylation	Pyrosequencing (cut-off for positive methylation: 10%)	73 cancer samples 17 normal breast samples* 15 paired normal breast samples	Higher *GSDME* promoter methylation in cancer compared to normal samples ($p < 0.001$) 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]
Kim *et al*, 2008[36]	Methylation	TaqMan-MSP (cut-off for positive methylation: 0.81)	34 cancer samples 13 paired normal breast samples 7 normal breast samples*	*GSDME* promoter: more often methylated in cancer compared to normal samples (*p value*: 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

Reference	Analysis	Technique	Number of samples	Result
	Gene expression	Real-time RT-PCR	1 cancer sample 1 paired normal breast sample 1 normal breast sample*	Lower *GSDME* expression in cancer compared to normal samples (paired: *p value* = 0.003; unpaired: *p value* = 0.002)
		Cancer Profiling Array	10 paired cancer – normal breast samples	Lower *GSDME* expression in 6/10 (60%) of cancer compared to normal samples
Thompson and Weigel, 1998[15]	Gene expression	Semi-quantitative RT-PCR	29 cancer samples: 15 ER+ 14 ER- 2 normal breast samples (ER-)	Lower *GSDME* expression in ER+ compared to ER- breast samples ($p < 0.001$)
COLORECTAL CANCER				
Ibrahim *et al*, 2019[28]	Methylation	TCGA - Infinium HumanMethylation450k array (22 CpGs in *GSDME*)	389 cancer samples 43 paired normal colon samples	Higher *GSDME* promoter (12/14 CpGs) methylation in cancer compared to normal samples (*p value* range: $1.7*10^{-16}$ – 0.025) Lower *GSDME* gene body methylation (5/6 CpGs) in cancer compared to normal samples (*p value range*: $8.3*10^{-9}$ – $4.5*10^{-3}$)
	Gene expression	TCGA - Agilent 244K Custom Gene Expression array	221 cancer samples 20 normal colon samples	No significant differences in *GSDME* expression between cancer samples and normal samples
		RNA-sequencing	437 cancer samples 39 normal colon samples	Mean *GSDME* expression: cancer samples: -0.46 (array); 5.45 (RNA-seq) normal samples: -3.18 (array), 5.8 (RNA-seq)
Yokomizo *et al*, 2012[39]	Methylation	qMSP	85 cancer samples 85 paired normal colorectal samples	*GSDME* methylated in: 29/85 (34%) cancer samples *No results for normal samples*
Kim *et al*, 2008[37]	Methylation	COBRA	10 cancer samples 9 paired normal colorectal samples	*GSDME* methylated in: 4/10 (40%) cancer samples 0/9 (0%) paired normal colorectal samples

Reference	Analysis	Technique	Number of samples	Result
		Bisulfite sequencing	5 cancer samples 10 paired normal colorectal samples	*GSDME* methylated in: 5/5 (100%) cancer samples 0/10 (0%) paired normal colorectal samples
		TaqMan-MSP (cut-off: 0.65)	100 cancer samples 100 paired normal colorectal samples 11 normal colorectal samples*	*GSDME* promoter: more often methylated in cancer compared to normal samples ($p < 0.001$) *GSDME* methylated in: 65/100 (65%) cancer samples 3/100 (3%) paired normal colorectal samples 1/11 (9%) normal colorectal samples*
	Gene expression	Real Time RT-PCR	5 cancer sample 5 paired normal breast sample 1 normal colon sample*	4/5 cancer samples reduced *GSDME* expression compared to pared normal samples *GSDME* expression in cancer 5x lower than in normal colon sample* (*p value*: 0.007)
GASTRIC CANCER				
Akino *et al*, 2006[35]	Methylation	COBRA Bisulfite sequencing (of region around TSS)	89 cancer samples 89 paired normal gastric samples	46/89 (52%) cancer samples: increased *GSDME* methylation 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	Methylated *GSDME*: Almost no *GSDME* expression (N = 5) Unmethylated *GSDME*: varying levels of *GSDME* expression (N = 5)
Kim *et al*, 2008[36]	Methylation	TaqMan-MSP (cut-off: 1)	31 cancer samples 11 paired normal gastric samples	*GSDME* methylated in: 17/31 (54%) cancer samples 1/11 (9%) paired normal gastric samples
ESOPHAGEAL CANCER				

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

*: 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