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Emerging links between endosomal pH and cancer

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Abstract

Extracellular acidification is a well-known driver of tumorigenesis that has been extensively studied. In contrast, the role of endosomal pH is novel and relatively unexplored. There is emerging evidence from a growing number of studies showing that the pH of endosomal compartments controls proliferation, migration, stemness, and sensitivity to chemo-radiation therapy in a variety of tumors. Endosomes are a crucial hub, mediating cellular communication with the external environment. By finely regulating the sorting and trafficking of vesicular cargo for degradation or recycling, endosomal pH determines the fate of plasma membrane proteins, lipids, and extracellular signals including growth factor receptors and their ligands. Several critical regulators of endosomal pH have been identified, including multiple isoforms of the family of electroneutral Na^+/H^+ exchangers (NHE) such as NHE6 and NHE9. Recent studies have shed light on molecular mechanisms linking endosomal pH to cancer malignancy. Manipulating endosomal pH by epigenetic reprogramming, small molecules, or nanoparticles may offer promising new options in cancer therapy. In this review, we summarize evidence linking endosomal pH to cancer, with a focus on the role of endosomal Na^+/H^+ exchangers and how they affect the prognosis of cancer patients, and also suggest how regulation of endosomal pH may be exploited to develop new cancer therapies.

Keywords

Sodium-Hydrogen Exchanger; NHE6; NHE9; RACK1; breast cancer; glioblastoma; chemoresistance; protons

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Introduction

Dysregulation of cellular pH is an established hallmark of malignancy [1–5]. In normal, differentiated cells, intracellular cytoplasmic pH is maintained at ~7.2 while extracellular pH stays at ~7.4. In contrast, cancer cells function in alkaline cytoplasmic pH conditions greater than 7.4 and hyperacidic extracellular pH of ~6.7–7.1 [1]. This reversal of the pH gradient between cytoplasmic and extracellular milieu promotes malignant phenotypes. High cytoplasmic pH is permissive to mitotic and meiotic re-entry in cells, and bypasses cell cycle checkpoints to promote cancer cell proliferation. Elevated cytoplasmic pH also helps cancer cells evade apoptosis since programmed cell death requires low cytoplasmic pH [6, 7]. In addition, the alkaline intracellular environment fosters genomic instability, which drives cancer evolution and therapeutic resistance by the generation of new subclones that establish the hierarchical organization of a tumor. Elevated H^+ extrusion has been proposed as a driver of the metabolic shift toward aerobic glycolysis, well known as the Warburg effect [8]. Furthermore, the acidic extracellular microenvironment in cancer promotes the expression of stem cell markers, angiogenic factors and hypoxia response factors enhancing tumor aggressiveness and angiogenic potential [9–11]. In malignant cells, a change in activity or localization of plasma membrane transporters favors tumor cell migration and metastasis. There are numerous reviews that discuss the oncogenic consequences of the transport of H^+, or their chemical equivalents HCO_3^- and OH^-, at the plasma membrane [1–3, 12].

In contrast to the extensive literature on the oncogenic role of extracellular pH, the impact of pH homeostasis within intracellular secretory or endo-lysosomal compartments on cancer growth, metastasis and drug resistance is only beginning to be appreciated. In the past decade, there have been several mechanistic studies that show the importance of endosomal pH in cancer phenotypes such as proliferation, migration, and stem cell-like properties [13–15]. Genomic data implicate key players in endosomal pH regulation in cancer survival prognosis and response to chemoradiation therapy. Proteins that play crucial roles in regulating the endosomal milieu and impact endosomal trafficking may be useful targets for the development of novel therapeutic agents. In this review, we evaluate the growing literature on the relevance of endosomal pH in cancer initiation, progression and metastasis, with particular emphasis on the role of organellar Na^+/H^+ exchangers.

Endosomes as a Hub in Cancer Signaling

The secretory pathway and the endo-lysosomal system handle the post-translational trafficking of proteins that exit the endoplasmic reticulum (ER) and the Golgi apparatus to and from the plasma membrane. This tubulo-vesicular network of compartments is responsible for the delivery, removal and inter-organellar shuttling of surface receptor proteins and their ligands that are critical to the dynamic process of cell-microenvironment communication. The last decade of cancer research has revealed cancer to be a collaborative entity that actively communicates with the surrounding microenvironment, rather than an autonomous chunk of multiplying cells. New players within the cancer microenvironment include cancer-associated fibroblasts and immune cells such as macrophages that communicate with each other and the tumor cells by paracrine signaling. Pre-clinical studies have begun to highlight potential, novel cancer therapies that target the critical

communications between cancer cells and their microenvironment. Due to their important role in regulation of cell-microenvironment communication, endosomes have emerged as an important hub in cancer cell signaling [16, 17].

At the plasma membrane, signaling is initiated by the binding of growth factors, cytokines, peptide agonists and other ligands to their cognate receptors to mediate cancer cell survival, self-renewal, proliferation or migration. Termination of signaling typically occurs by endocytosis, with pH-dependent dissociation of ligands from their receptors occurring within the acidic milieu of the endosomal lumen, followed by delivery of membranes to the lysosome for receptor degradation. Alternatively, the receptor-ligand complex may be retrieved from early endosome for rapid return to the cell surface, or sorted to perinuclear recycling compartments for slower delivery to the plasma membrane [18]. Thus, endosomal trafficking could decide the fate of cancer cells by termination or prolongation of oncogenic signaling. In the case of the epidermal growth factor receptor (EGFR), one of many receptor tyrosine kinases (RTK) that play a central role in carcinogenesis, the magnitude of signaling has been clearly linked to endocytic fate by extensive studies [16, 17].

Endosomes may themselves serve as signaling platforms for the recruitment of scaffolding proteins by bringing the activated G protein coupled receptors (GPCR) or RTK in contact with localized pools of adaptors or effector molecules to control both the duration and spatial distribution of signaling. For example, endosomal AKT kinase phosphorylates GSK3β via the APPL1 scaffold, whereas plasma membrane AKT preferentially activates the TOR pathway through phosphorylation of TSC2 [19]. Altered endocytic trafficking in cancer cells is crucial for the maintenance and metastasis of tumors and has been comprehensively reviewed [17, 18, 20].

The Critical Role of pH in Endosomal Function

Given the importance of the endosome as a hub for trafficking, recycling and turnover of cellular cargo in tumor cells, endosomal pH emerges as a critical, but a largely under-explored topic of investigation that could reveal new therapeutic opportunities in cancer. For normal function, the secretory pathway and endo-lysosomal system absolutely requires a pH gradient of increasing acidification of the luminal compartment from the Golgi through the *trans* Golgi network (pH 6.7 to 6), and from early (pH ~6.5) to late (pH ~5.5) endosomes and lysosomes (pH ~4.5)[21]. A shift in the tightly regulated compartmental pH disrupts a wide range of downstream processes such as protein sorting, quality control and degradation, activation of proteases, and exocytosis [13, 22]. In addition to these direct roles, endosomal pH is also inextricably linked to the flux of other ions such as Na^+, K^+, Cl^-, Cu^{2+}, Zn^{2+} and Ca^{2+} that are critical for signaling and biogenesis of enzymes. Movement of these ions in and out of the endosomes occurs through various ion transporters, pumps and channels that may be powered by the proton gradient established by the V-type H^+-ATPase, or regulated by luminal pH.

The machinery underlying vesicle budding, targeting and fusion is orchestrated by small GTPases that appear to use endosomal pH as cues for critical sorting decisions. Thus, the recruitment of Arf1 and Arf6 small GTPases, and their guanine nucleotide exchange factor

(GEF) ARNO, to the endosomal membrane is pH-dependent and can be uncoupled by disruption of the endosomal pH gradient [23]. However, the molecular mechanism for sensing luminal pH and transmitting this information to the cytoplasmic face of the endosome remains largely mysterious. Marshansky and colleagues have proposed that the membrane-embedded VATPase a2 subunit may act as pH sensor, through histidine-rich intra-endosomal loops or termini [24] and that interactions between the V-ATPase subunit and GEFs may modulate GDP/GTP exchange activity [25].

In the following sections, we will discuss the key players involved in the intricate regulation of endosomal pH and how they may contribute to cancer initiation, progression, metastasis, and patient outcome.

Regulators of Endosomal pH

A plethora of ion transporters and channels have been implicated in the regulation of endosomal pH throughout different, endosomal compartments. Among these, the best known are the V-type H^+-ATPase [22], and isoforms of Chloride transporters (CLCs) [22, 26], and Sodium-Hydrogen (Na^+/H^+) exchangers (NHEs) [27] that collaborate to finely tune compartmental pH as depicted in Figure 1.

(i) Proton pump:

The V-type H^+-ATPase is an evolutionarily conserved pump that couples ATP hydrolysis within the large cytosolic domain to the uphill, transmembrane movement of protons into the lumen. However, the movement of positively charged protons generates an opposing membrane potential that will cause the V-ATPase to stall, hindering the build up of protons required to acidify the compartmental lumen. The electrical potential can be shunted by outward movement of cations, such as K^+, or inward movement of anions, such as Cl^-, allowing the formation of a pH (i.e., H^+ chemical) gradient. Increased expression and relocation of the V-ATPase to the plasma membrane in cancer cells has been linked to oncogenic phenotypes including Warburg effect, autophagy, drug resistance and regulation of signaling pathways, and has been reviewed [28, 29].

(ii) Chloride transporters:

Members of the CLC family comprise Cl^- channels as well as Cl^-/H^+ exchangers, including isoforms CLC3–7 that shunt the electrical potential generated by the VATPase to acidify the endo-lysosomal lumen [30]. Cl^- flux is critical for endocytosis in renal tissue [31] where defects in CLC-5 (gene name *CLCN5*) underlie Dent's disease characterized by proximal tubule dysfunction and low molecular weight proteinuria [32, 33]. Consistent with their essential role in pH regulation, loss of function mutations in the chloride transporter isoform, CLC-7 (gene name *CLCN7*), phenocopy V-ATPase defects in the fatal disorder, osteopetrosis in which defective acidification by osteoclasts results in failure to remodel bone [34]. Thus, H^+ and Cl^- fluxes within the endo-lysosomal system are tightly intertwined, and they both play critical roles in cargo trafficking, vesicular transport and compartment maturation [35]. The CLC3 isoform has been implicated in several cancer

types, including breast, cervical and prostate cancer, where it promotes drug resistance and metastatic phenotypes [36].

(iii) Cation Proton Exchangers:

$Na^+(K^+)/H^+$ exchange is remarkably rapid, with transport rates of 1,500 ions per second [37] so that small changes in activity can cause large pH shifts within the confines of the endosomal lumen. Several members of the SLC9A/NHE family are major contributors to pH homeostasis within secretory and vesicular compartments. Based on evolutionary origin and sequence similarities, the family is subdivided into two major groups: the plasma membrane and intracellular subgroups [38]. The plasma membrane subgroup consists of isoforms NHE1–5, which are coupled to the Na^+ gradient established by the Na^+,K^+-ATPase, whereas members of the intracellular subgroup, NHE6–9, are driven by the vesicular V-type H^+ pump. Although belonging to the plasma membrane subgroup, both NHE3 and NHE5 have significant presence in endocytic compartments where they exchange luminal Na^+ ions for cytoplasmic H^+, contributing to luminal acidification [39, 40]. The existence of this additional machinery for luminal acidification, along with V-ATPases, may explain rapid re-acidification of synaptic vesicles upon endocytosis with experimentally measured time constant of ~0.4s [41] or 4–5s [42] in cultured hippocampal neurons. NHE3-mediated acidification was shown to be important for receptor-mediated endocytosis of albumin in the first part of the endocytic pathway in a renal epithelial cell model [40]. Similarly, NHE5 is a potent acidifier of recycling endosomes in rat pheochromocytoma PC12 cells, and attenuation of NHE5 expression via shRNA decreased the steady state level of Tropomyosin Receptor Kinase A (TrkA) on the plasma membrane [43].

Intracellular members of the NHE superfamily are distributed in the Golgi (NHE8), *trans*-Golgi network (NHE7), recycling (NHE9) and early (NHE6) endosomes. Compartment-specific localization of these transporters appears to be linked to their binding partners that include members of the secretory carrier membrane protein (SCAMP), caveolins and receptor for activated protein C kinase (RACK1) [44–46]. Unlike the strict Na^+-selectivity of the plasma membrane group, intracellular NHE can transport K^+ as well as Na^+ ions [47, 48], thereby taking advantage of the abundance (~140 mM) of K^+ in the cytoplasm. Thus, the prevailing pH and K^+ gradients across the vesicular membrane favor luminal H^+ efflux in exchange for K^+ loading by NHE6 and NHE9 (Figure 1). This is consistent with the majority of studies, which report that NHE6, NHE7 and NHE9 alkalinize the compartmental lumen [13, 47, 49–53], in contrast to the “plasma membrane” subtype isoforms, NHE3 and NHE5 that acidify endosomal lumen [40].

Endosomal pH in Cancer Progression and Metastasis

Cancer is a multi-step process which begins with the accumulation of 2–3 driver mutations [54] en route to full transformation [55]. For metastasis to occur, primary tumor cells locally invade the surrounding tissue, then intravasate into the blood stream to eventually extravasate to initiate secondary tumors, often at a distant site [56]. Considering that most cancer patients succumb to this final step in cancer progression, there is an urgent need to understand the biology underlying metastasis and to uncover molecular targets to slow or

prevent metastasis. There is evidence that organellar pH is altered during cell transformation: for example, fibroblasts transformed with *ras* oncogene displayed more alkaline lysosomes [57]. It has long been appreciated that the papillomavirus protein E5 mediates cellular transformation by binding to, and inhibiting the V-ATPase, consequently slowing endosomal acidification, neutralizing Golgi pH and alkalinizing lysosomes [58]. Intriguingly, extracellular vesicles known as large oncosomes are shed from primary glioblastoma cells. These oncosomes, carrying VATPase V1G1 subunit and homeobox proteins, require active V-ATPase activity to mediate cell-cell signaling and tumor reprogramming of the non-neoplastic environment [59].

Acidic metabolic by-products are produced and accumulate due to the hypoxic tumor microenvironment. Along with the evolutionary selection of cancer-driving mutations, the acidic tumor microenvironment favors and drives cancer progression [1], particularly in local invasion and metastasis [5], genetic instability [60–64], cancer stem cells [9], epigenetic alterations [64], proliferation [1, 65], and survival [66–68]. Acidic extracellular pH between 6.4 and 6.8 was found to increase lysosome size and promote anterograde trafficking to the cell periphery resulting in increased secretion of proteolytic enzymes [69, 70]. Interestingly, the anterograde trafficking of the lysosomes is inhibited with treatments with broad Na^+/H^+ Exchanger (NHE) inhibitors, 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) and troglitazone, suggesting a potential involvement of endosomal NHE isoforms, NHE6 and NHE9 [69] and consistent with recent pre-clinical and clinical studies implicating these endosomal NHE isoforms to local invasion [13] and metastatic potential in cancer [71].

Molecular mechanisms linking endosomal pH to cancer

In recent years, significant progress has been made towards elucidating the cellular processes and signaling pathways that link endosomal pH, and particularly dysregulated expression or activity of intracellular NHE isoforms to oncogenesis and chemoresistance. These studies are invaluable in revealing potential drug synergies and new molecular targets that could be developed for cancer therapy. Table 1 summarizes studies on intracellular NHE in cancer, with emerging mechanistic insights described below.

(i) Hypoxia-driven drug resistance:

Hyperacidification of the endosomal lumen promotes the partitioning of weakly basic drugs into endosomes and confers multidrug resistance (MDR) by preventing drug accumulation in the target cell or organelle, and also by facilitating exocytosis of the drug [72–74]. This type of resistance to therapy is intensified in an intratumoral hypoxic environment [75]. Examples of weakly basic drugs include the anthracyclines - for example, doxorubicin (Dox), mitoxantrone (Mtx), and daunorubicin (Dnr) - that exert their chemotherapeutic effect by damaging DNA; therefore, the effectiveness of such cytotoxic drugs requires accumulation inside the nucleus. In addition to genetic alterations, therapeutic resistance to anthracycline drugs can arise from physical and chemical changes in the tumor microenvironment, such as extracellular pH, that prevent accumulation of the drugs in cancer cells [76–78]. Lucien et al. showed that hypoxic conditions of 1% oxygen cause hyperacidification of the endosomal lumen [52, 79]. This caused trapping of Dox in the

endosomes (Figure 2A), diverting the drug from the nucleus and resulting in four to eight-fold higher drug resistance in breast cancer MDA-MB-231 and fibrosarcoma HT-1080 tumor cells [52]. Hypoxia-induced drug resistance was reversed when the cells were treated with chloroquine or V-ATPase inhibitor (Bafilomycin A1) to alkalinize the endosomal compartments, consistent with a causal role for endosomal pH in drug resistance. The number of endosomes and lysosomes did not change in hypoxia, consistent with a role for altered luminal pH in drug resistance, rather than changes in the relative population of endo-lysosomal compartments. Lucien et al. found that silencing NHE6 but not NHE9 increased endosomal drug sequestration, even under normoxia. We note that co-localization of Dox with transferrin within 20 min of uptake is consistent with the early endosomal function of NHE6 and suggest that Dox sequestration is crucially affected by the pH of the early endosomal compartment.

The hypoxia-induced reduction in endosomal pH was traced to a relocalization of NHE6 from endosomes to plasma membrane, without alteration in NHE6 transcript and protein level [52]. Activation of protein kinase C (PKC) by hypoxia recruited the scaffold protein RACK1 (receptor for activated C Kinase 1), previously shown to bind to the C-terminal cytoplasmic domain of NHE6 [44]. Accordingly, treatment of cells with the $NHE6^{527-588}$ peptide from the RACK1 binding region was effective in disrupting the interaction between RACK1 and NHE6, to abrogate Dox sequestration in hypoxic condition. The potential for $NHE6^{527-588}$ peptide as a therapeutic agent in enhancing Dox efficacy was demonstrated in a human cancer cell ex-ovo chorioallantoic membrane (CAM) xenograft model in live chicken embryos [52]. This study presented a previously unknown role of NHE6 in cancer and suggested a novel, molecular mechanism for hypoxia-driven, endosomal pH-dependent drug resistance [79].

(ii) Response to chemoradiation therapy:

Consistent with high amplification of *SLC9A9* (Figure 3), NHE9 was found to be a prognostic predictor for poor survival in esophageal squamous cell carcinoma [80]. Esophageal cancer is the ninth most common cancer in the world based on the collected data from 2018, causing 572,034 new cases and 508,585 deaths worldwide [81]. The highest rates of incidence for esophageal cancer occur in eastern Asia, southern Africa, and eastern Africa [81]. Esophageal cancers can be divided into two main histological subtypes: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EA). Despite the discoveries of new therapeutic agents for ESCCs, patient prognosis has remained poor with the five-year survival rate of less than 20% [82]. Using overexpression and shRNA-mediated knockdown strategies, Chen et al. [83] demonstrated a role for NHE9 in increased resistance to apoptosis induced by chemotherapy agents, including cisplatin (DNA-damaging) and vinorelbine (microtubule inhibitor), and X-ray radiation. The increased chemoradiation resistance with NHE9 expression was also shown in xenograft model in mice. The pro-survival function of NHE9 was attributed to the interaction with RACK1 through a conserved region in the C-terminal tail (Figure 2B). RACK1 is known to suppress apoptosis by increasing p-Akt and p-Src, and Chen et al. showed that NHE9 increases anti-apoptotic and pro-survival pathways such as p-GSK3b, Bcl-2, β-catenin, p-Akt and p-Src to inhibit cleavage of PARP or Caspase-3. The NHE9-RACK1 interaction was

weakened following chemoradiation treatment, presumably activating pro-survival pathways from RACK1, although the mechanism linking NHE9 activity and endosomal pH to RACK1 in mediating pro-survival pathways remains to be determined.

(iii) Aggressive tumor growth and migration:

Repositories of patient data are a useful starting point to focus on the role of individual gene loci in specific cancers and their molecular subtypes. Glioblastoma patients with elevated NHE9 expression were associated with decreased survival and increased resistance to chemoradiation therapy by Kaplan-Meier analysis of data from The Cancer Genome Atlas (TCGA). Kondapalli et al. noted that NHE9 transcript was elevated in glioblastoma patients, especially in the aggressive mesenchymal subtype, compared to non-malignant neural stem cells [13]. Using patient derived glioblastoma cell lines they demonstrated that up regulated expression of NHE9 significantly alkalinized endosomal lumen, to pH ~6.5. Elevated endosomal pH blocked lysosomal degradation of EGFR, increased persistence of EGFR on the tumor cell surface and prolonged downstream signaling by p-AKT and p-ERK, driving malignant phenotypes such as increased tumor proliferation and migration (Figure 2C). Similarly, high levels of NHE9 expression correlated with increased metastasis and worse prognosis in colorectal cancer patients, where a positive correlation with EGFR signaling was also noted [71]. Post-translational oncogenic activation of EGFR was dependent on alkalinization of endosomal pH because autism-associated loss-of-function mutations, S438P and L236S, failed to phenocopy wild type NHE9 in glioblastoma cells. The increased aggressiveness of tumor due to higher NHE9 level was confirmed in orthotopic patient-derived xenograft (PDX) models [13].

The study by Kondapalli and co-workers provided a molecular explanation of the role of NHE9 in a human malignancy, uncovering an unexplored link between endosomal pH dysregulation and glioblastoma. However, the underlying reason for hyperexpression of NHE9 in brain tumors remained unanswered. Zubieta et al. followed up by demonstrating that microRNA-135a with a seed sequence targeting 3'-UTR of *SLC9A9* was down regulated in glioblastoma cell lines, compared to normal human brain tissue, resulting in increased NHE9 expression [84]. Functional restoration of miR-135a by ectopic expression in GBM cell lines resulted in down regulation of *SLC9A9* transcript, acidification of endosomal pH and decreased GBM cell proliferation and migration. Colocalization of intracellular EGFR with the lysosomal marker LAMP1 was enhanced by miR-135a, suggesting that decreased endosomal pH due to down regulation of NHE9 facilitated lysosomal degradation of EGFR.

The importance of endosomal pH in the regulation of receptor trafficking and degradation in tumor glioma cells was also observed in the context of NHE5, with intriguingly opposite effects to that of NHE9, highlighting the exquisite specificity of NHE isoforms in targeting distinct trafficking pathways. The expression of NHE5 is greatly enriched in neurons, and although not detected in glia-rich regions, was found to be elevated in the rat C6 glioma cell line where it partially colocalized with some endosomal markers, including TfR and Rab11 [14]. Fan *et al.* showed that NHE5 knockdown and treatment with the V-ATPase inhibitor bafilomycin had independent and additive effects in alkalinizing TfR-positive recycling

endosomal compartments by small, but significant amounts of ~0.2–0.3 pH units. Surface levels of the receptor tyrosine kinase MET decreased with NHE5 knockdown, with concomitant reduction in downstream activation by hepatocyte growth factor (HGF) of PI3K/Akt pathway and activities of critical regulators of cytoskeleton remodelers such as the Rho family small GTPase protein, Rac1, and CDC42 (Figure 2D). Decreased association of Rac1 with the leading edge of C6 rat glioma was attributed to decreased cell migration in vitro and polarity in NHE5 knockdown cells [14]. In addition, Fan et al. showed that NHE5 knockdown abrogated the recycling of MET receptor, but not Tfn receptors, whereas the endosome recycling inhibitor, primaquine, inhibited recycling of both MET and Tfn. Subsequent study from Fan et al. further showed that NHE5 increases the total level of EGFR and MET while abrogating the downstream signaling proteins' activation in C6 rat glioma cells. The authors also demonstrated that NHE5 regulates intracellular trafficking of integrin 1β to enhance attachment and migration of cancer cells (Figure 4D) [85]. These studies provide interesting perspective to the field of endosomal pH and its contribution to cancer aggressiveness. Further validation of the role of NHE5 in multiple, patient-derived glioma cells would be useful in clarifying differences in the roles of NHE isoforms in glioma [86], and provide better insights on how to utilize this knowledge to benefit patients in clinic.

(iv) Cancer initiation and stemness:

For the past decades, the capacity of cancer stem cells (CSCs) to initiate new tumor has been extensively appreciated [55]. Further investigation also revealed that CSCs are major players in recurrence [87], metastasis[88], and chemoradiation resistance [89] of cancer. There has been growing interest in defining molecular factors that regulate CSCs to exploit them as potential therapeutic targets [87, 90]. *In vitro* evaluation of self-renewal and cancer initiation capacity of CSCs is performed by sphere- or colony-formation assay. shRNA-mediated knockdown of NHE9 significantly decreased the sphere-forming ability [71]. In addition, forced differentiation of patient-derived glioblastoma cells by serum treatment decreased the level of NHE9 [13], indicating that alkalinization of endosomal compartment via NHE9 could play a critical role in stemness maintenance.

Although localized primarily in the *trans*-Golgi network, NHE7 also dynamically traffics between the endosomes and the plasma membrane, regulating the luminal pH of organelles [91]. Onishi *et al*. expressed NHE7 in a triple negative breast cancer cell line, MDA-MB-231, and observed increased growth, invasiveness, and colony formation in soft agar, consistent with a potential role for NHE7 in cancer growth, metastasis, and tumorigenesis [92]. Although this is an intriguing result, more mechanistic studies in multiple cell lines of different types of malignancy still remain to be done to establish clear relevance to cancer patients.

Phylogenetic analysis identified NHE8 as an intracellular NHE, albeit clustering in a distinct subgroup, separate from NHE6, NHE7, and NHE9 [38]. Indeed, when expressed in CHO and HeLa cells, NHE8 localizes to *mid*- to *trans*- Golgi, and to a lesser extent in the endosome [47]. However, NHE8 also localizes to the apical side of the proximal tubule of the kidney and the intestine, playing a critical role in gastric mucosal integrity and protection

against bacterial infection [93, 94]. Gene disruption of NHE8 causes mice to become more susceptible to spontaneous colitis, dysbiosis, and increased epithelial cell proliferation [93]. Xu *et al.* showed that NHE8 is highly expressed at the colonic epithelial lining of normal tissue, but is absent in colorectal cancer tissue from patients [95]. NHE8 knockout mice had 10-fold higher incidence of tumor in an inflammation-associated colon cancer model. The authors attributed this difference to increased number of Lrg5-expressing cells and elevated Wnt/β-catenin activation. Lgr5-expressing cells have been shown to be the cell of origin for colorectal cancer, maintaining cellular hierarchy and driving metastasis [96, 97]. These findings justify the need for more mechanistic studies on how NHE8 contributes to Lgr5 levels and Wnt/β-catenin signaling.

(v) Cancer Metastasis:

It is well known that the acidic microenvironment resulting from hypoxia, increased interstitial pressure and the accumulation of acid metabolites is associated with regional and distant metastasis. For example, acidic pH was found to promote lung metastases of human melanoma cells in the athymic mouse model through the activation of extracellular proteases, including cathepsins [98]. In human metastatic breast cancer cells, the presence of large acidic vesicles was associated with invasion, consistent with a requirement for endosomal acidification for cathepsin D maturation and activation [99]. It remains to be determined if excessive acidification resulting from loss of NHE6 expression or increased V-ATPase activity in early endosomes promotes secretion of extracellular proteases. On the other hand, alkalinization of recycling endosomes has been associated with cancer cell migration and metastasis. Using intracranial xenograft models of glioblastoma, NHE9 expression increased tumor infiltration in the mouse brain [13]. A histological analysis of colorectal cancer in 6 patients revealed elevated expression of NHE9 in liver metastases [71]. More work remains to be done to determine if endosomal pH dysregulation is associated with specific organ metastases.

Genomic Profiling of eNHE in Cancer

(i) Genome-Wide Association Studies (GWAS):

The link between NHE9 (*SLC9A9*) and cancer initially arose out of large-scale genomic studies on patient samples, ranging from single nucleotide polymorphism (SNP) analysis to expression profiling. In a comprehensive molecular characterization of human colon and rectal cancer, whole-genome sequencing of 276 samples revealed *SLC9A9* was one of 7 most frequent targets of mutation in hypermutated cancers [100]. Picelli *et al.* investigated genome-wide linkage between chromosomal regions and familial susceptibility to colorectal cancer in 30 families with strong history of colorectal cancer and reported that the strongest linkage with colorectal cancer susceptibility was found in chromosome 3q, which included *SLC9A9* among the top 20 cancer susceptibility genes [101]. Transcriptional down regulation of *SLC9A9* was associated with tumor progression into hormone-refractory stage in prostate cancer [102], potentially through NHE9-mediated post-translational effects on surface expression of the androgen receptor. These studies have firmly established NHE9 as a strong candidate for cancer susceptibility in cancers of the gastrointestinal tract where it is considered a driver gene, as well as brain, breast, prostate and ovarian cancers. More

recently, mechanistic studies have begun to provide insight into oncogenic pathways altered by NHE9, described ahead.

(ii) Expression Profiling in Cancer Databases:

Analysis of patient data in The Cancer Genome Atlas (TCGA) datasets revealed *SLC9A9* and *SLC9A6* expressions are relatively evenly distributed across 32 cancers, depicted in the order of increasing expression in Figure 3. These findings are consistent with ubiquitous expression of *SLC9A9* and *SLC9A6* across all tissues, with generally higher expression of *SLC9A6* than *SLC9A9* [103]. Robust expressions of *SLC9A9* and *SLC9A6* in the brain have been previously reported in the literature in the context of normal and psychiatric conditions consistent with high levels of expression in glioma patients [103–105]. Gene amplification events were more common in *SLC9A9*, especially in cervical, ovarian and head and neck cancers, and in lung squamous cell carcinoma (Figure 3). In contrast, majority of patients across all cancer types had shallow deletions in *SLC9A6*. For *SLC9A9*, gene amplification was observed in 14% of esophageal squamous cell carcinoma, 9% of cervical squamous cell carcinoma, and 6% of non-small cell lung cancer patients. On the other hand, the most frequently observed genetic alterations in *SLC9A6* were gene mutations, reported in 7% of endometrial carcinoma, 3% of melanoma, and 2% of non-small cell lung cancer patients. Thus, genetic alterations in *SLC9A9* and *SLC9A6* are cancer type-specific and isoform-specific, which should be taken into consideration when trying to target endosomal NHE in cancer.

(iii) Mutational Analysis:

Recurring somatic mutations in cancer have been proposed to confer selective advantage, such as charge-changing mutations in pH sensing [106]. Despite the cancer-specific enrichment of mutation incidents in *SLC9A6*, the overall somatic mutation frequencies for both *SLC9A9* and *SLC9A6* were comparable at 1.5% and 1.1%, respectively (Figure 4). In *SLC9A9*, the most frequent mutation, S355L, was present in four uterine endometrioid carcinoma and one tubular stomach adenocarcinoma patients; Interestingly, according to the dbPTM database (http://dbptm.mbc.nctu.edu.tw/index.php), S355 residue is a potential phosphorylation site [107], which may influence the function and trafficking of NHE9. Other post-translational modification sites that were mutated are N96Y, an N-glycosylation site [108, 109], and Y631*, a tyrosine phosphorylation site [107]. Mutation at R468 in the predicted cytosolic domain of NHE6 was observed in four patients: R468Q in one head and neck squamous cell carcinoma patient, R468* in one rectal adenocarcinoma patient and one uterine endometrioid carcinoma patient, and R468L in one cutaneous melanoma patient. Overall, of gene alterations in *SLC9A9*, 81% were missense mutations, 14% truncating mutations, and 4% fusion events while, in *SLC9A6*, 84% were missense mutations and 16% were truncations (Figure 4). While the pathogenicity of missense mutations remains to be assessed, truncating mutations within the conserved, transmembrane NHE coding region are likely to be detrimental to protein function and stability.

(iv) MicroRNA:

Genomic profiling of cancer samples has consistently revealed an interesting link between NHE6 and microRNAs (miRNA). MiRNAs are small (18–25 nucleotides), single stranded,

evolutionarily-conserved non-coding RNA molecules that target complementary mRNA for degradation in order to negatively regulate gene expression [110]. Alterations in expression of various miRNAs have been noted in multiple malignancies where they significantly correlate with patient outcomes [111]. However, their heterogeneous tissue expression and lack of specificity make it difficult to classify individual miRNA as tumor suppressors or oncogenes resulting in conflicting reports in the literature. Despite these challenges, *SLC9A6* has emerged as a target gene of significantly altered microRNAs in neoplastic samples. miR-196a is one of the most significantly over-expressed miRNAs in cervical cancer where it targets *SLC9A6* along with well-known genes involved with development and cellular remodeling, *HOXC8* and *HOXA7*[111]. Another frequently altered miR-196 family member in cancer is miR-196b which is down regulated in cervical cancer [112] and overexpressed in recurrent epithelial ovarian cancer cells [113], driving malignant growth and invasiveness. *SLC9A6* is one of the top target genes of mir-196b. These findings suggest that the absence of NHE6 is related to tumor progression. This hypothesis is supported by a mechanistic study of *SLC9A6* in breast cancer cells, described ahead.

Targeting endosomal pH in cancer

(i) eNHE inhibitors:

While there are currently available inhibitors that effectively target members of the plasma membrane NHE subtype, specific and selective inhibitors or activators against the endosomal NHE are lacking and urgently needed. It is possible to exploit the wide range of drugs already targeted against plasma membrane NHE isoforms as a starting point in inhibitor screening and development. Proof-of-principle experiments have illustrated the potential utility of targeting endosomal NHE: for example, EIPA was shown to effectively inhibit NHE9 and increase drug efficacy of anti-EGFR inhibitor erlotinib and reduce tumorsphere formation in gliobastoma models [13]. EIPA has also been used and effective to inhibit NHE7 and abrogate proton-loading mechanism in endocytosis [114].

(ii) Gene modifiers:

Alternatively, genetic approaches could be used to elevate or ablate NHE transcripts. Epigenetic modifiers such as histone deacetylase inhibitors were used to regulate endosomal pH by enhancing expression of NHE6 transcript in an Alzheimer disease model [115, 116]. Poly (beta-amino ester) (PBAE) nanoparticles that harbor positively charged amine groups capable of binding nucleic acids such as siRNA could be used as a non-viral delivery method to target isoform-selective mRNAs. Recent success has been achieved in using PBAE nanoparticles for combinatorial siRNA delivery to selectively target brain tumor cells, while sparing stroma cells in the microenvironment [117, 118].

(iii) Nanoparticles:

Kondapalli and co-workers recently exploited the endocytosis-enhancing ability of NHE9 to target macrophages with gold nanoparticles [119]. Selective uptake of these particles in NHE9-overexpressing glioblastoma cells by receptor-mediated endocytosis rendered them susceptible to near-infra red radiation, resulting in apoptotic death of tumor cells. Furthermore, the ability of macrophages loaded with gold nanoparticles to cross the blood

brain barrier points to the potential for nanoparticle based therapy to target brain tumors. Interestingly, nanoparticles could directly regulated luminal pH along the endo-lysosomal pathway: for example, acidic nanoparticles contain FDA-approved poly (DL-lactide-co-glycolide) (PLGA), which is hydrolyzed rapidly to lactic acid in the endosomal lumen releasing protons [120, 121], offering an innovative way to acidify hyper-alkaline pH.

(iv) Weak acid/base drugs and peptides:

Another approach is to repurpose drugs that partition into the acidic endosomal lumen and mildly alkalinize the pH. Examples of such drugs include bepridril and amiodarone, already in clinical use as calcium antagonists, used to correct endosomal pH in Alzheimer disease models of amyloid peptide processing [122]. Lucien et al. described the use of short peptides to compete against RACK1 binding of NHE6 and reverse endosomal acidification [52, 79]. Such innovative approaches arise by exploiting newly found understanding of the basic mechanisms that contribute to tumor growth and drug resistance.

Remaining Challenges

Although significant advances have been made in understanding how the luminal pH of the secretory and endo-lysosomal pathway is regulated, new players are still being identified. For example, a recently discovered acid-activated chloride leak channel in endosomes may serve to limit vesicular acidification [123] although its role, if any, in driving oncogenic change has not been explored. The precise roles and interactions between endosomal NHE isoforms also remain to be determined. These differences may be cell type and therefore, cancer subtype specific. Surprisingly, both alkalinization as well as acidification of endosomes due to increased NHE9 and NHE5 activity respectively, has been linked to glioma. Early and recycling endosome pH may play distinct and potentially opposing roles in promoting cancer growth, invasion and chemoresistance, which need to be resolved. It is possible that a deviation from optimal pH regardless of the direction could be critical in cancer malignancies, emphasizing the importance of precisely tuned luminal pH. Distribution of intracellular NHE isoforms is likely to be cell and tissue specific, illustrating the need for more investigations with appropriate cell lines and primary cells of the tissue of interest in order to make the most relevant observations. Consistent with their similar, yet distinct subcellular localizations, NHE6 and NHE9 also have overlapping, yet non-redundant effects on plasma membrane retention of cell surface receptors and their ligands. A non-biased proteomic analysis could help define these receptor pools, which could add specificity to future therapeutic efforts.

The oncogenic role of binding partners of the C-terminal tail of endosomal NHE proteins still remained to be explored. Plasma membrane NHE isoforms have been heavily studied in this regard, exemplified by studies showing that the C-terminal tail of NHE1 regulates trafficking and activity of the membrane embedded transporter domain by protein binding and phosphorylation [124, 125]. Similar investigations on the C-terminal tail of endosomal NHE isoforms and their binding partners and post-translational modifications are urgently needed to provide insights on their trafficking and transport function. Such additional

knowledge will enable us to understand the physiological regulatory mechanisms of endosomal NHEs and how those processes can go awry, leading to malignant phenotypes.

Conclusion

Hyperacidified extracellular pH in cancer has been highly scrutinized through mechanistic studies on plasma membrane transporters such as NHE1. However, changes in intracellular pH accompanying malignancy are just beginning to be appreciated. Endosomes have been recognized as a critical signaling hub in tumor cells and a busy way station for the trafficking, degradation, and recycling of oncogenic receptors and other cargo. Each of these functions is critically impacted by endosomal pH, with profound effects on patient survival prognosis. More studies are needed to distinguish between the oncogenic role of pH in different endosomal populations, and to track dynamic localization of endosomal NHE isoforms in malignant disease settings for insights on underlying mechanisms. We suggest that endosomal pH drives malignant phenotypes ranging from unregulated growth and migration to chemo-radiation resistance, justifying the need for further studies on endosomal pH regulators in various cancer types. Development of endosomal NHE-specific inhibitors and activators with minimal off-target effect will be critical for translating findings from bench to bedside.

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Abbreviations

EGFR	epidermal growth factor receptor
ESCC	esophageal squamous cell carcinoma
GBM	glioblastoma
GPCR	G-protein coupled receptor
GEF	guanine nucleotide exchange factor
NHE	Sodium Hydrogen Exchanger
RTK	receptor tyrosine kinase
Tfn	transferrin

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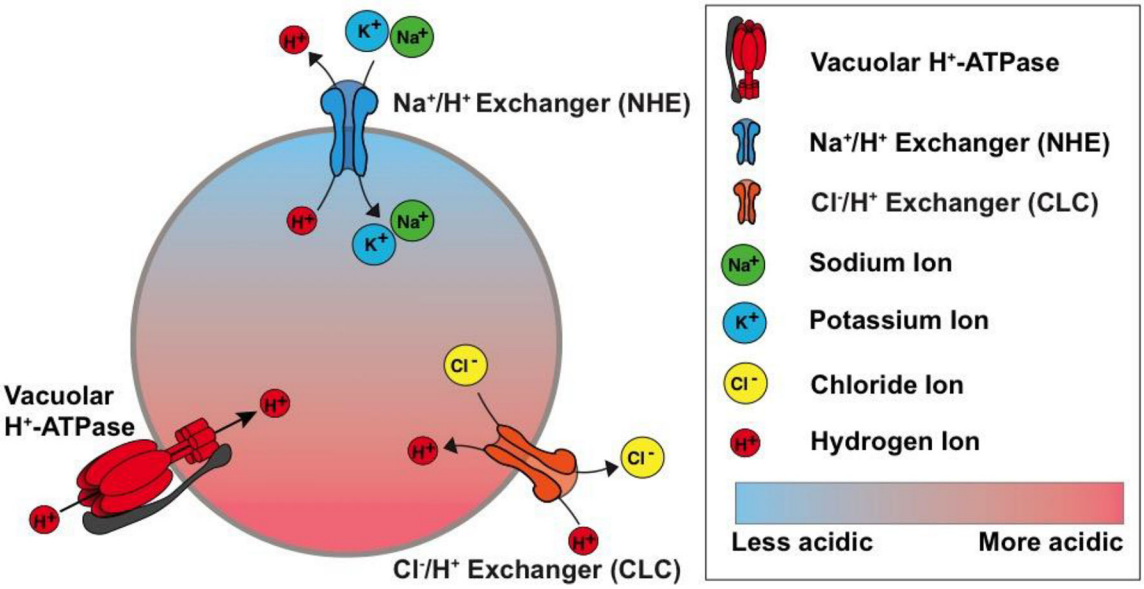

Figure 1. Endosomal pH is determined by a balance of proton pump and leak mechanisms.

The concerted action of the V-type H^+-ATPase and members of the CLC family of H^+/Cl^- exchangers acidifies the lumen of endosomes. Intracellular members of the NHE family of Na^+/H^+ exchangers finely tune endosomal pH by leaking protons in exchange for Na^+ and K^+.

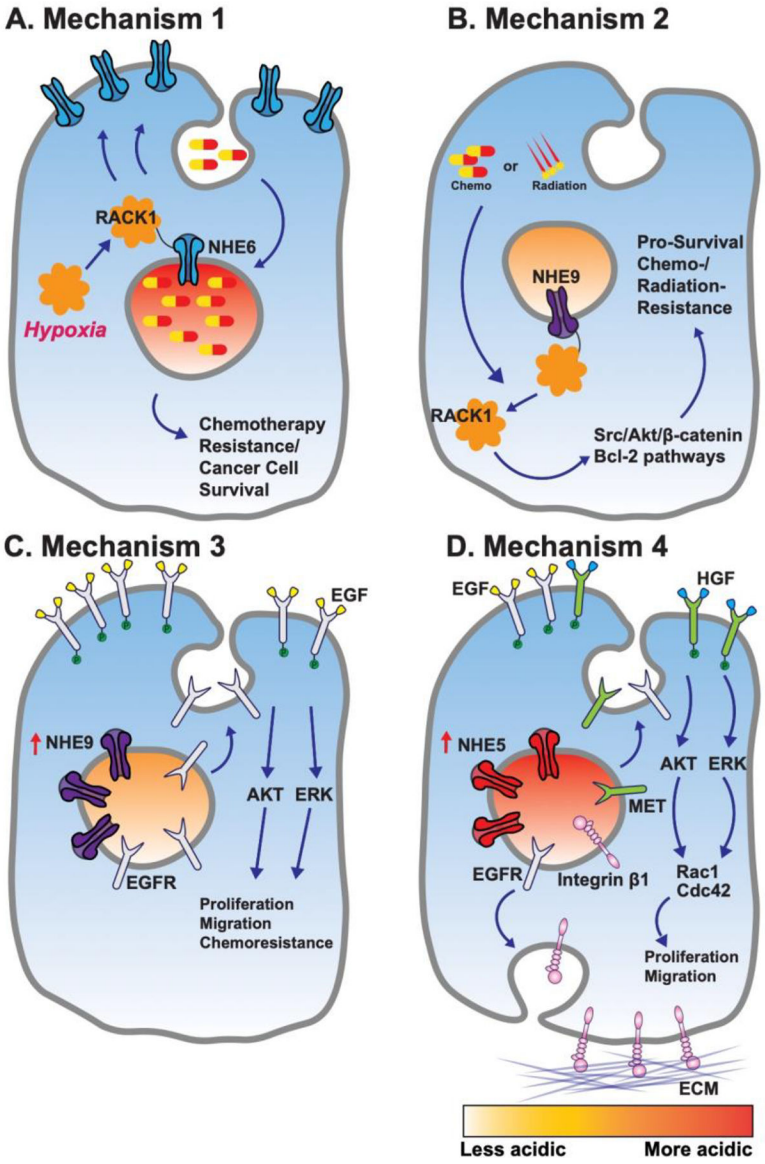

Figure 2. Molecular mechanisms proposed for NHE isoforms in cancer.

A. In response to hypoxia, RACK1 mediates the translocation of NHE6 from the endosomes of breast cancer cells to the plasma membrane. This results in acidification of endosomal compartment and sequestration of weakly basic drugs such as doxorubicin. B. Upon treatment of cancer cells with chemotherapy and radiation, RACK1 is released from the C-terminal tail of NHE9 to facilitate downstream activation of Src/Akt/β-Catenin and Bcl-2 pathway, resulting in chemoradiation resistance. C. In glioblastoma, NHE9 is highly expressed, resulting in alkalinization of the endosomal lumen. As a result, oncogenic receptors such as EGFR escape degradation and are recycled back to the plasma membrane to drive tumor growth, migration and chemoresistance. D. NHE5 level is elevated in rat C5 glioblastoma cell line, where it acidifies the endosomal compartment. High levels of NHE5 are proposed to increase EGFR, MET, and integrinβ on the cell surface to drive proliferation and migration of cancer cells.

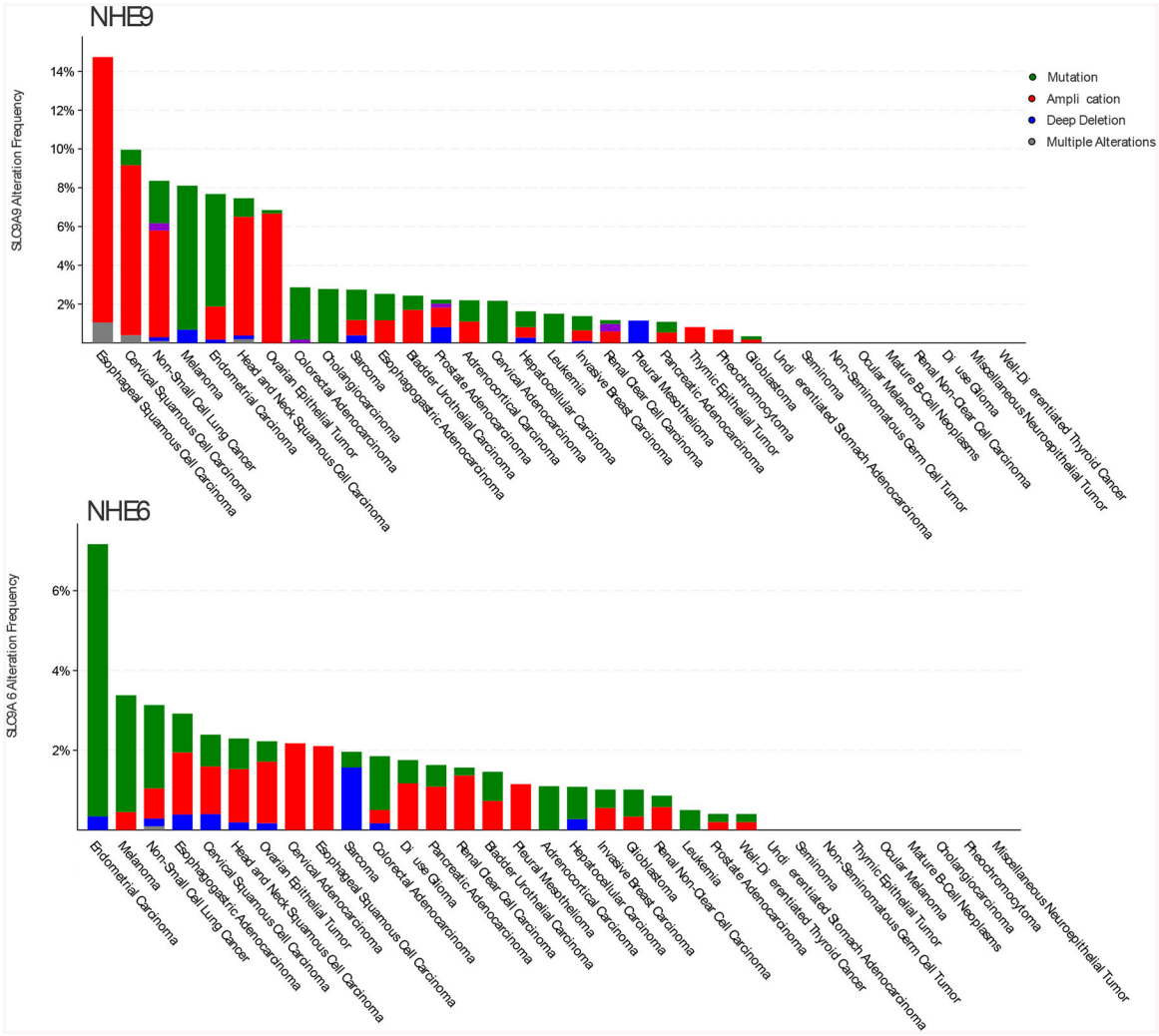

Figure 3. Gene alterations in endosomal NHE across cancer subtypes.

Alterations are shown for NHE9 (top) and NHE6 (bottom) and comprise mutation, amplification, and deep deletion. Amplifications may include focal gene amplifications or larger chromosome parts such as whole chromosome gains. Combined RNA-Seq V2 and mutational data from 10,953 patients included in the TCGA PanCancer study across 32 tumor types. Dataset is from cBioPortal.

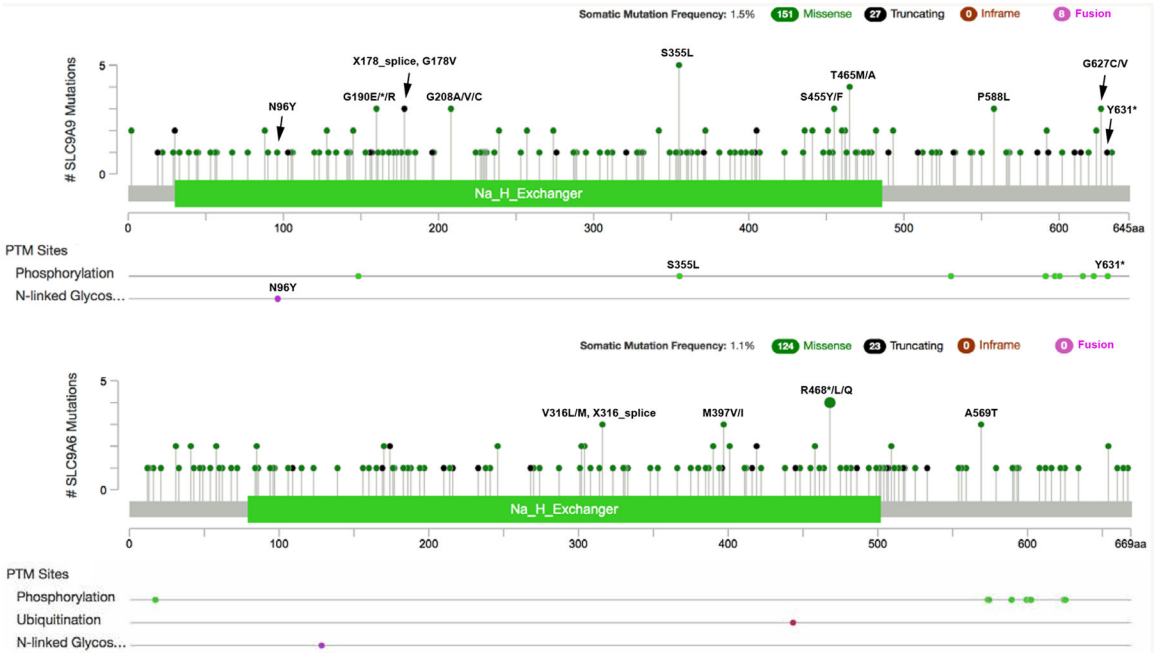

Figure 4. Somatic mutations in endosomal NHE found in cancer.

Lollipop representation of the frequency and types of somatic mutations found in NHE9 gene (*SLC9A9*, top) and NHE6 gene (*SLC9A6*, bottom). Predicted PTM (post-translational modification) sites are also indicated. The data shows *SLC9A9* gene harbors mutations on two phosphorylation sites (S355L and Y631*) and one N-linked glycosylation site (N96Y) in cancer.

Table 1.

Summary of alterations in intracellular NHE isoforms reported in cancer types.

Cancer type	Intracellular NHE Protein	Expression in Cancer	Study	Model(s)	Summary	Citation
GBM (Glioma)	NHE9	↑	Kondapalli KC et al. *Nat. Comm.* 2015	1. Patient-derved human glioblastoma cells 2. Orthotopic, intracranial mouse xenograft	Up-regulation of NHE9 expression alkalinizes endosomal compartment and results in EGFR stabilization on the cell surface in glioblastoma cells, driving proliferation and migration	[13]
	NHE5	↑	SH Fan et al. *Mol. Biol. Cell.* 2016	Rat Glioma cell line: C6	Up-regulation of NHE5 expression acidifies endosomes and facilitates recycling of MET to drive cell migration in Rat glioma cell line	[14]
		↑	Kurata T et al. Clin. Exp. Metastasis. 2019		Comparison study between NHE1 and NHE5 knockdown shows unique role of NHE5 in integrin stabilization and MET/EGFR signaling, resulting in defects in migration and proliferation	[85]
Breast Cancer	NHE7	?	Onish I et al. *Oncol. Rep.* 2012	MDA-MB-231 cell line	Overexpression of NHE1 and NHE7 in triple negative breast cancer cell line, MDA-MB-231, shows the role of NHE7 in cancer cell growth, migration, and colony formation	[92]
	NHE6	Translocation to the plasma membrane in hypoxic condition	Lucien F et al. *Nat. Comm.* 2017	MDA-MB231 cell line	Under hypoxic cancer microenvironment, NHE6 is mislocalized to the plasma membrane, depleting the pool of NHE6 protein in the endosomal vesicles. This results in acidification of endosomal compartment in which weak-base chemotherapy drugs are trapped.	[52]
Fibrosarcoma				HT-1080 cell line		[52]
Colorectal Cancer	NHE8	↓	Xu H et al. *Cell Mol Gastroenterol. Hepatol.* 2019	1. Chemically induced colorectal cancer model in NHE8 knockout mice 2. HT-29 human colorecetal cancer line	Azoxymethane/dextran sodium sulfate colon cancer model with NHE8 knockout background reveals a previously unexlplored role of NHE8 in suppressing colo cancer initiation.	[95]
Esophageal Squamous Cell Carcinoma	NHE9	↑	Chen J et al. *Oncotarget* 2015	Eca190 and KYSE30 cell lines	NHE9 is upregulated in esophageal squamouse cell carinoma patients. NHE9 also induces chemo- and radiation-therapy resistance by decreasing pro-apoptotic signaling pathway via losing its direct interaction with RACK1 scaffold protein and activating Src/Akt/β-catenin and Bcl-2	[83]