4. Objective II. Study the molecular mechanism of apoptosis resistance on prolong TRAIL treatment.

4.1. Background of the study

TRAIL is well known for its unique ability in inducing apoptosis in a tissue specific manner unlike the other family members of TNF superfamily. However, there is a limitation in this ability of TRAIL as it does not induce apoptosis in all kinds of tumor cells[56,158]. Even repeated administration makes the TRAIL sensitive cells resistant to TRAIL induced apoptosis. Previously we have reported that resistance towards TRAIL-induced apoptosis can be acquired at any stage of signaling pathway, starting from the death receptor (DR) to the expression of apoptotic inhibitors[97,134]. Mutation occurred at the death receptors causing loss of function or by blocking the receptors from binding to the TRAIL can confer resistance to the apoptotic pathways[61,63]. Dysfunctioning of protein Fas-associated death domain (FADD) and absence of different caspases can even produce resistance to cell death [63]. Recruitment of cFLIP to disc, overexpression of Bcl-2 and Bcl-XL, dysfunctioning of Bax, accumulation apoptotic inhibitors and reduced release of second mitochondria-derived activator of caspases (Smac/Diablo) from the mitochondria to the cytosol are some of the main ways of TRAIL resistance[64,161]. Resistance is even offered by Inhibitors of apoptosis (IAP) proteins, Six1 expression and O-glycosylation of receptors[63,162]. Lastly TRAIL not only activates apoptosis but also pro-survival factor NF-κB activation of which may counteract apoptosis[99].In the present study we are trying to understand the mechanism of inherently acquired TRAIL resistant in TNB cancer cell line MDA-MB-231.

It has been already reported that EMT plays an important role in TRAIL resistances, role of migration and invasion have shown to be critical factors regulating TRAIL resistance in TNBC cell lines[103]. One of the essential epithelial transmembrane proteins regulating

EMT is CDH1[104], a homophilic calcium-dependent cell adhesion molecule[105]. Loss of tumor suppresser CDH1 plays a crucial role in dysfunction of cell-cell adhesion. Therefore, the loss of cellular integrity will lead to local metastasis and invasion of the tumor cells[104]. Reduced cell-cell adhesion and increased cell-EMC adhesion is one of the definitive markers of tumor cells. In gastric cancer high density of collagen helps in integrin-mediated cell–ECM interactions and loss of membranous E-cadherin[106]. Homotypic interaction of E-cadherin regulated actin and membrane dynamics of extracellular matrix during early stage of cell-cell adhesion[107]. Earlier it has been reported that loss of CDH1 causes aberrant cell cycle entry of post-mitotic neurons, thereby leading to apoptosis[108]. Proteolysis of CDH1 leads to accumulation of cyclin B1 that in turn promotes apoptosis[109]. Studies have shown ability of TRAIL to form death receptor complex relies on EC1 extracellular domain of E-cadherin. E-cadherin/α-catenin mediated linkage of actin cytoskeleton plays a vital role in activation of DISC[110]. Embodiment of DR4 and DR5 in CDH1 adhesion structures, utilizes the structural ability of actomyosin contractility for effective assembly of DISC components[111].

4.2. Results

4.2.1. Selection of inherently hrTRAIL resistant cells from TRAIL sensitive MDA-MB-231 cell line.

The in-vitro sensitivity of triple negative cell line MDA-MB-231 to rhTRAIL was already reported in some studies. In the present study, we have established a TRAIL resistant triple negative breast cancer cell line MB231/TRAIL by exposing the TRAIL sensitive MDA-MB-231 with high dose of rhTRAIL (800µg/ml), where the IC50 of normal cells was 280µg/ml. The surviving population of cells were grown in culture media. Positive rhTRAIL resistant cells were tested with trypan blue dye-exclusion test and MTT assay with 500ng/ml rhTRAIL treatment (Fig. 1a, 1b). Furthermore, Staining of rhTRAIL treated MDA-MB-231 and MB231/TRAIL cells with showed some typical morphological changes that are associated with apoptosis only in normal MDA-MB-231, but not in

MB231/TRAILMembrane blebbing, which represents early apoptosis, increased significantly after 6 hrs of rhTRAIL treatment in control MDA-MB-231 in comparison to MB231/TRAIL ($P<0.05$) (Fig. 1c, 1e). Similarly, late apoptosis, as indicated by the presence of EtBr-stained red nucleus, showed an increase on rhTRAIL treatment in sensitive cell line but absent in resistant cells ($P<0.05$) (Fig. 1d, 1f).

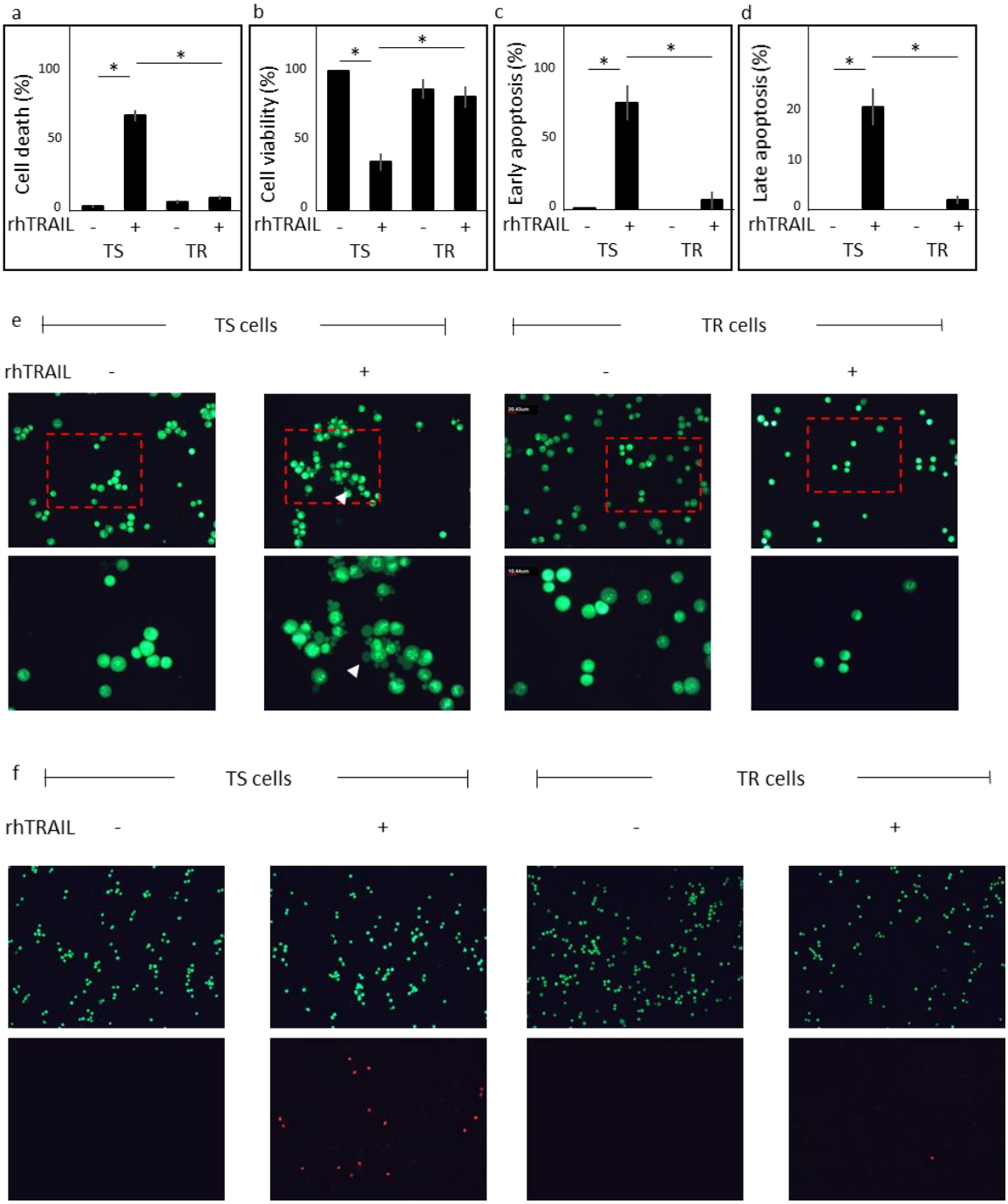

Figure 4.1. *MDA-MB-231 TR cell population show resistance towards TRAIL mediated cytotoxicity on exposure to rhTRAIL. Inherent TRAIL resistant (TR) cells were selected*

from a population of TRAIL sensitive (TS) MDA-MB-231 cells using 800 ng/ml (IC99) rhTRAIL. The TR cells and TS cells were maintained in cell culture with 500ng/ml rhTRAIL and mock treatment, respectively, on every alternate passage. Next, both TS cells and TR cells were treated with rhTRAIL (500ng/ml) for 24hrs. Cytotoxicity was measured by (a) trypan blue assay and (b) MTT assay. AO/EtBr staining was used to measure (c) early apoptosis and (d) late apoptosis after 6-hrs of rhTRAIL (500ng/ml) treatment in TS and TR cells. (e) Representative image of cells showing membrane blebbing (arrow) after staining with AO dye. (f) Representative image of cells showing EtBr stained cells after AO/EtBr staining. All experiments were repeated three times. Representative experiment has been shown in the figure.

4.2.2. Differentially expressed genes among rhTRAIL resistant and sensitive population were analysed via DAVID software

For better understanding of relationship between differentially gene regulation and inherent TRAIL resistance in MDA-MB-231 cells, microarray analysis was performed using TR cells where parental TS cells were treated as control cells. Out of analysed 32,045 probes, 6,598 probes were found to be significantly different between the TR and TS cells. 6,598 probes consisted of 5,247 genes having NM ID out of which we observed 4,907 number of unique DEGs.

Next, to identify the differentially expressed genes (DEGs) responsible for rhTRAIL resistance in MDA-MB-231 cells, microarray analysis was performed using TR cells where parental TS cells were used as control. Out of 32,045 probes that were analyzed, 6,598 probes were found to be significantly different between the TR and TS cells. 6,598 probes consisted of 5,247 genes having NM ID out of which we observed 4,907 number of unique DEGs. The distribution of DEGs is shown in Fig 2a. Next, the genes were shortlisted based on fold change of greater than 2 and less than -2 to obtain a set of 216 DEGs. Out of the 216 DEG genes, we observed 83 genes to be upregulated and 133 genes to be downregulated in TR cells in comparison to control TS cells (Fig. 2b, Table 4.1, 4.2). 216 DEGs that were analysed using DAVID (Annotation, Visualization, and Integrated Discovery) for functional annotations. DAVID provided us with various Gene Ontology (GO) enrichment analysis of our DEGs, where we found 40 significant enriched biological process (BP), 9 enriched cellular components (CC) and 9 enriched molecular function

(MF) GO terms present in TR cells(Fig. 2c, 2d, Table 4.3). KEGG pathway analysis of 216 DEGs via DAVID showed 7 significant pathways: hsa04610: Complement and coagulation cascades, hsa04971: Gastric acid secretion, hsa04750: Inflammatory mediator regulation of TRP channels, hsa04725: Cholinergic synapse, hsa04670: Leukocyte transendothelial migration, hsa04514: Cell adhesion molecules (CAMs) and hsa04060: Cytokine-cytokine receptor interaction (Fig. 2e, Table 4.4).

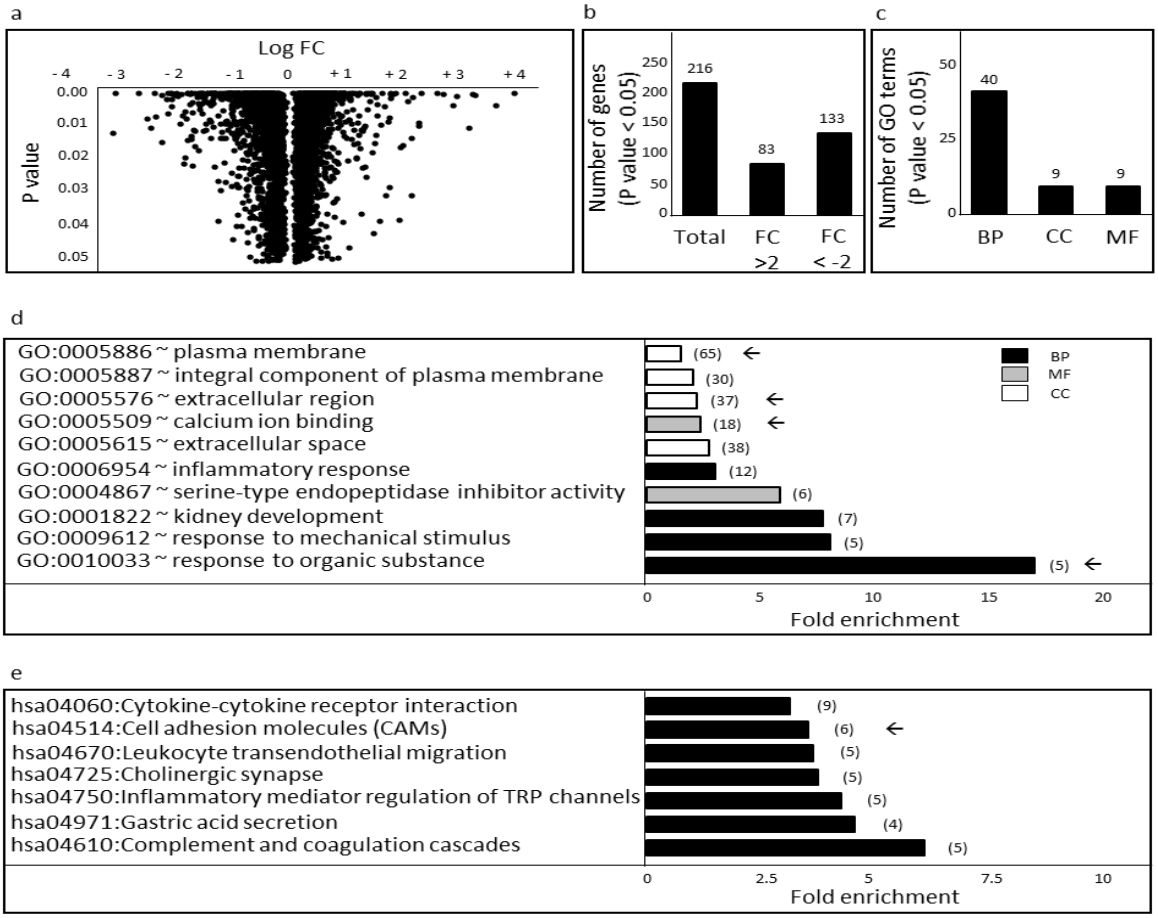

Figure 4.2. *Analysis of microarray data for identification and functional characterization of candidate differentially expressed genes (DEGs) in MDA-MB-231 TR cells. Microarray experiment was carried out in duplicate for TS and TR cells. (a) The volcano plot shows 4907 significant DEGs that are present in TR cells in comparison to TS cells. (b) The distribution of significant DEGs (216 genes) in TR cells that show a fold change (FC) of greater than 2 and less than -2. (c) The number of significant GO terms obtained through DAVID by analyzing the DEGs shown in figure 2(b) belonging to biological process (BP), cellular component (CC) and molecular function (MF). (d) The top 10 significant GO terms and (e) significant KEGG pathways obtained by analyzing the DEGs shown in figure 2(b). The number in parenthesis beside each bar indicates the gene count corresponding*

to the respective GO and KEGG pathway category. Arrow indicates that CDH1 belongs to that particular category.

Table 4.1: Genes that are upregulated in TR cells compared to TS cells

GeneSym	GenbankAcce	GeneName
CLDN11	NM_005602	claudin 11
SERPINB	NM_002575	serpin peptidase inhibitor, clade B (ovalbumin), member 2
SPOCK1	NM_004598	sparc/osteonectin, cwcv and kazal-like domains proteoglycan
MAGEA	NM_153488	melanoma antigen family A, 2B
LPHN2	NM_00129770	latrophilin 2
CYP24A	NM_000782	cytochrome P450, family 24, subfamily A, polypeptide 1
MAGEA	NM_005367	melanoma antigen family A, 12
SERPIN A9	NM_175739	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 9
GPC6	NM_005708	glypican 6
CSAG1	NM_153478	chondrosarcoma associated gene 1
GCKR	NM_001486	glucokinase (hexokinase 4) regulator
CADM1	NM_00130104	cell adhesion molecule 1
ARHGAP	NM_020809	Rho GTPase activating protein 20
LYPD5	NM_182573	LY6/PLAUR domain containing 5
AFP	NM_001134	alpha-fetoprotein
CAMK2	NM_172082	calcium/calmodulin-dependent protein kinase II beta
TNFSF15	NM_005118	tumor necrosis factor (ligand) superfamily, member 15
RNF17	NM_031277	ring finger protein 17
PSG5	NM_00113001	pregnancy specific beta-1-glycoprotein 5
NPPB	NM_002521	natriuretic peptide B
RAB38	NM_022337	RAB38, member RAS oncogene family
KCNK2	NM_001017424	potassium channel, two pore domain subfamily K, member 2
HRASLS	NM_020386	HRAS-like suppressor
ANXA10	NM_007193	annexin A10
KISS1	NM_002256	KiSS-1 metastasis-suppressor
TMEM74	NM_153015	transmembrane protein 74
PSG8	NM_182707	pregnancy specific beta-1-glycoprotein 8
VCX2	NM_016378	variable charge, X-linked 2
HTR1F	NM_000866	5-hydroxytryptamine (serotonin) receptor 1F, G protein-coupled
TMEM45	NM_138788	transmembrane protein 45B
CXCR4	NM_00100854	chemokine (C-X-C motif) receptor 4
ANTXRL	NM_00127868	anthrax toxin receptor-like
C6orf99	NM_00130283	chromosome 6 open reading frame 99
SLC14A1	NM_001146037	solute carrier family 14 (urea transporter), member 1 (Kidd blood group)

GeneSym	GenbankAcce	GeneName
CYP2J2	NM_000775	cytochrome P450, family 2, subfamily J, polypeptide 2
MAST4	NM_198828	microtubule associated serine/threonine kinase family
BMPER	NM_133468	BMP binding endothelial regulator
FAM177	NM_207468	family with sequence similarity 177, member B
EFR3B	NM_014971	EFR3 homolog B (S. cerevisiae)
KRT33B	NM_002279	keratin 33B, type I
COL1A2	NM_000089	collagen, type I, alpha 2
KAL1	NM_000216	Kallmann syndrome 1 sequence
CCDC85	NM_00108043	coiled-coil domain containing 85A
HS3ST3B	NM_006041	heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1
NGFR	NM_002507	nerve growth factor receptor
CLDN2	NM_00117109	claudin 2
C4BPB	NM_000716	complement component 4 binding protein, beta
LINGO2	NM_152570	leucine rich repeat and Ig domain containing 2
RGS4	NM_005613	regulator of G-protein signaling 4
CD69	NM_001781	CD69 molecule
CA9	NM_001216	carbonic anhydrase IX
CYB5RL	NM_00103167	cytochrome b5 reductase-like
CCND2	NM_001759	cyclin D2
CD3G	NM_000073	CD3g molecule, gamma (CD3-TCR complex)
PLA2G10	NM_003561	phospholipase A2, group X
PDZK1IP	NM_005764	PDZK1 interacting protein 1
FAM110	NM_00107771	family with sequence similarity 110, member C
TNIK	NM_015028	TRAF2 and NCK interacting kinase
GJB3	NM_024009	gap junction protein, beta 3, 31kDa
SERINC2	NM_178865	serine incorporator 2
HAS2	NM_005328	hyaluronan synthase 2
HEMGN	NM_018437	hemogen
CLEC17	NM_00120411	C-type lectin domain family 17, member A
SLC35F1	NM_00102985	solute carrier family 35, member F1
ZFYVE2	NM_00117265	zinc finger, FYVE domain containing 28
STC1	NM_003155	stanniocalcin 1
WDR78	NM_207014	WD repeat domain 78
SPRED3	NM_00104252	sprouty-related, EVH1 domain containing 3
RARRES	NM_002889	retinoic acid receptor responder (tazarotene induced) 2
HCLS1	NM_005335	hematopoietic cell-specific Lyn substrate 1
FMOD	NM_002023	fibromodulin
LOX	NM_002317	lysyl oxidase
UTRN	NM_007124	utrophin
RNF152	NM_173557	ring finger protein 152
COL12A	NM_004370	collagen, type XII, alpha 1
SCAF1	NM_021228	SR-related CTD-associated factor 1
FAM196	NM_00112989	family with sequence similarity 196, member B
FAM133	NM_173698	family with sequence similarity 133, member A
MROH8	NM_213631	maestro heat-like repeat family member 8

GeneSym	GenbankAcce	GeneName
HHIP	NM_022475	hedgehog interacting protein
IL17A	NM_002190	interleukin 17A
SAMD12	NM_00110167	sterile alpha motif domain containing 12
CCNA1	NM_003914	cyclin A1

Table 4.2: Genes that are downregulated in TR cells compared to TS cells

GeneSymbol	GenbankAccession	GeneName
IL21R	NM_181078	interleukin 21 receptor
GDF15	NM_004864	growth differentiation factor 15
NUPR1	NM_001042483	nuclear protein, transcriptional regulator, 1
PECAM1	NM_000442	platelet/endothelial cell adhesion molecule 1
PADI4	NM_012387	peptidyl arginine deiminase, type IV
GGT5	NM_001099781	gamma-glutamyltransferase 5
LAIR2	NM_002288	leukocyte-associated immunoglobulin-like receptor 2
PRSS45	NM_199183	protease, serine, 45
SLCO4C1	NM_180991	solute carrier organic anion transporter family, member 4C1
NFE2	NM_006163	nuclear factor, erythroid 2
NMNAT3	NM_178177	nicotinamide nucleotide adenylyltransferase 3
INHBB	NM_002193	inhibin, beta B
FSTL5	NM_020116	follistatin-like 5
IFI44L	NM_006820	interferon-induced protein 44-like
BDKRB2	NM_000623	bradykinin receptor B2
APCDD1	NM_153000	adenomatosis polyposis coli down-regulated 1
ELF3	NM_004433	E74-like factor 3 (ets domain transcription factor, epithelial-specific)
PLEKHG1	NM_001029884	pleckstrin homology domain containing, family G (with RhoGef domain) member 1
LEFTY1	NM_020997	left-right determination factor 1
SLC13A3	NM_001011554	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3
IL2RB	NM_000878	interleukin 2 receptor, beta
VAV3	NM_006113	vav 3 guanine nucleotide exchange factor
SLPI	NM_003064	secretory leukocyte peptidase inhibitor
RGS9	NM_001165933	regulator of G-protein signaling 9
PAEP	NM_002571	progestagen-associated endometrial protein
KIF12	NM_138424	kinesin family member 12

GeneSymbol	GenbankAccession	GeneName
C7orf65	NM_001123065	chromosome 7 open reading frame 65
BDKRB1	NM_000710	bradykinin receptor B1
RAB37	NM_175738	RAB37, member RAS oncogene family
CEL	NM_001807	carboxyl ester lipase
EGR2	NM_000399	early growth response 2
SIRPG	NM_018556	signal-regulatory protein gamma
CREB3L3	NM_032607	cAMP responsive element binding protein 3-like 3
WNT5A	NM_003392	wingless-type MMTV integration site family, member 5A
ACKR3	NM_020311	atypical chemokine receptor 3
S100P	NM_005980	S100 calcium binding protein P
PADI3	NM_016233	peptidyl arginine deiminase, type III
SLC26A10	NM_133489	solute carrier family 26, member 10
DGKK	NM_001013742	diacylglycerol kinase, kappa
CFH	NM_001014975	complement factor H
CRIP1	NM_001311	cysteine-rich protein 1 (intestinal)
TET2	NM_017628	tet methylcytosine dioxygenase 2
LDLRAD4	NM_181482	low density lipoprotein receptor class A domain containing 4
PCSK9	NM_174936	proprotein convertase subtilisin/kexin type 9
CDH1	NM_004360	cadherin 1, type 1, E-cadherin (epithelial)
SMAD6	NM_005585	SMAD family member 6
DACH1	NM_080759	dachshund family transcription factor 1
FABP3	NM_004102	fatty acid binding protein 3, muscle and heart
GREB1	NM_014668	growth regulation by estrogen in breast cancer 1
DLL1	NM_005618	delta-like 1 (Drosophila)
AGT	NM_000029	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
TENM2	NM_001122679	teneurin transmembrane protein 2
SORBS2	NM_021069	sorbin and SH3 domain containing 2
ACTG2	NM_001615	actin, gamma 2, smooth muscle, enteric
GORAB	NM_001146039	golgin, RAB6-interacting
TMEM98	NM_015544	transmembrane protein 98
KCNQ1	NM_000218	potassium channel, voltage gated KQT-like subfamily Q, member 1
PHGDH	NM_006623	phosphoglycerate dehydrogenase
CCR1	NM_001295	chemokine (C-C motif) receptor 1

GeneSymbol	GenbankAccession	GeneName
XKR3	NM_175878	XK, Kell blood group complex subunit-related family, member 3
ENG	NM_000118	endoglin
CRH	NM_000756	corticotropin releasing hormone
SHISA9	NM_001145204	shisa family member 9
PLCB1	NM_015192	phospholipase C, beta 1 (phosphoinositide-specific)
RBP1	NM_002899	retinol binding protein 1, cellular
SOST	NM_025237	sclerostin
KRT86	NM_002284	keratin 86, type II
FAM110D	NM_024869	family with sequence similarity 110, member D
COLEC12	NM_130386	collectin sub-family member 12
RUNX3	NM_001031680	runt-related transcription factor 3
KLRC1	NM_007328	killer cell lectin-like receptor subfamily C, member 1
OR2F2	NM_001004685	olfactory receptor, family 2, subfamily F, member 2
SYT17	NM_016524	synaptotagmin XVII
PLEKHS1	NM_024889	pleckstrin homology domain containing, family S member 1
LRRC48	NM_031294	leucine rich repeat containing 48
PDE7B	NM_018945	phosphodiesterase 7B
GPR20	NM_005293	G protein-coupled receptor 20
UBD	NM_006398	ubiquitin D
SULF2	NM_018837	sulfatase 2
KLRC4	NM_013431	killer cell lectin-like receptor subfamily C, member 4
FAM65C	NM_001290268	family with sequence similarity 65, member C
ARMC12	NM_145028	armadillo repeat containing 12
PREX1	NM_020820	phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1
DDIT4	NM_019058	DNA-damage-inducible transcript 4
STRA6	NM_001199042	stimulated by retinoic acid 6
DZIP1	NM_198968	DAZ interacting zinc finger protein 1
MYBPH	NM_004997	myosin binding protein H
WNK2	NM_006648	WNK lysine deficient protein kinase 2
HEATR4	NM_203309	HEAT repeat containing 4
AUTS2	NM_015570	autism susceptibility candidate 2
BCHE	NM_000055	butyrylcholinesterase

GeneSymbol	GenbankAccession	GeneName
CEACAM1	NM_001712	carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)
NTN1	NM_004822	netrin 1
MADCAM1	NM_130760	mucosal vascular addressin cell adhesion molecule 1
NOX1	NM_013955	NADPH oxidase 1
WISP2	NM_003881	WNT1 inducible signaling pathway protein 2
FOXS1	NM_004118	forkhead box S1
BMF	NM_001003940	Bcl2 modifying factor
FBXO32	NM_058229	F-box protein 32
SLC16A1	NM_001166496	solute carrier family 16 (monocarboxylate transporter), member 1
IER5L	NM_203434	immediate early response 5-like
FBXL16	NM_153350	F-box and leucine-rich repeat protein 16
C1orf111	NM_182581	chromosome 1 open reading frame 111
SPRY1	NM_199327	sprouty homolog 1, antagonist of FGF signaling (Drosophila)
LIPG	NM_006033	lipase, endothelial
MEGF6	NM_001409	multiple EGF-like-domains 6
SUSD2	NM_019601	sushi domain containing 2
CAPN3	NM_173087	calpain 3, (p94)
RGN	NM_152869	regucalcin
C8orf4	NM_020130	chromosome 8 open reading frame 4
C9orf139	NM_207511	chromosome 9 open reading frame 139
HMGCS1	NM_002130	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)
SNAP91	NM_001256717	synaptosomal-associated protein, 91kDa
ABCG1	NM_207627	ATP-binding cassette, sub-family G (WHITE), member 1
FPR1	NM_002029	formyl peptide receptor 1
NTN4	NM_021229	netrin 4
TMEM61	NM_182532	transmembrane protein 61
KCNJ12	NM_021012	potassium channel, inwardly rectifying subfamily J, member 12
TRIM22	NM_006074	tripartite motif containing 22
CCNG2	NM_004354	cyclin G2
SEL1L3	NM_015187	sel-1 suppressor of lin-12-like 3 (C. elegans)
KYNU	NM_001032998	kynureninase

GeneSymbol	GenbankAccession	GeneName
NEURL3	NM_001285486	neuralized E3 ubiquitin protein ligase 3
KDM7A	NM_030647	lysine (K)-specific demethylase 7A
CPE	NM_001873	carboxypeptidase E
ALDOC	NM_005165	aldolase C, fructose-bisphosphate
MATN3	NM_002381	matrilin 3
ALOX5AP	NM_001629	arachidonate 5-lipoxygenase-activating protein
HOXC4	NM_014620	homeobox C4
METTL7A	NM_014033	methyltransferase like 7A
RPGRIP1	NM_020366	retinitis pigmentosa GTPase regulator interacting protein 1
SERPINA5	NM_000624	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5
IQCA1	NM_024726	IQ motif containing with AAA domain 1

Table 4.3: Top 10 significant GO terms based on 216 differentially expressed genes.

Sr. No	GO terms	GO category	Gene Count	Fold Enrichment	Gene Name
1	GO:0010033~response to organic substance	BP	5	16.8	WNT5A, CRIP1, S100P, CDH1, ABCG1
2	GO:0009612~response to mechanical stimulus	BP	5	8	INHBB, SOST, BDKRB1, BDKRB2, KCNK2
3	GO:0001822~kidney development	BP	7	7.6	SULF2, AGT, RGN, PCSK9, STRA6, HAS2, TET2
4	GO:0004867~serine-type endopeptidase inhibitor activity	MF	6	5.8	SERPINA9, AGT, SERPINA5, SERPINB2, SLPI, SPOCK1
5	GO:0006954~inflammatory response	BP	12	3	GGT5, IL17A, RARRES2, ELF3, CXCR4, CCR1, NOX1, CRH, FPR1, BDKRB1, NGFR, BDKRB2
6	GO:0005615~extracellular space	CC	38	2.7	WNT5A, FMOD,

					TNFSF15, SPOCK1, KRT33B, KISS1, ACTG2, IL17A, WISP2, SERPINA9, AGT, SERPINA5, GPC6, CFH, COL12A1, PCSK9, LOX, KRT86, C4BPB, LEFTY1, INHBB, CEL, AFP, BMPER, SOST, CPE, SULF2, PECAM1, COL1A2, CRH, FABP3, LIPG, SERPINB2, SLPI, NPPB, STC1, GDF15, ENG
7	GO:0005509~calcium ion binding	MF	18	2.3	MATN3, S100P, PLA2G10, PADI3, CDH1, DLL1, SPOCK1, PADI4, CAPN3, SULF2, ANXA10, CD69, TENM2, FSTL5, RGN, PLCB1, MEGF6, SYT17
8	GO:0005576~extracellular region	CC	37	2.2	WNT5A, FMOD, RARRES2, CDH1, KISS1, IL17A, BCHE, AGT, SERPINA5, FSTL5, CFH, RGN, COL12A1, HHIP, LOX, MATN3, PLA2G10, LAIR2, NTN4, C4BPB, DLL1,

					NTN1, INHBB, CEL, BMPER, PSG8, SOST, PSG5, COL1A2, CRH, LIPG, SERPINB2, NPPB, PAEP, NGFR, GDF15, MEGF6
9	GO:0005887~integral component of plasma membrane	CC	30	2	CADM1, CCR1, FPR1, TNFSF15, BDKRB1, BDKRB2, KCNJ12, APCDD1, SLC26A10, SLC16A1, CD69, GPC6, HHIP, GPR20, HTR1F, CEACAM1, IL2RB, SLCO4C1, CD3G, DLL1, KCNK2, ABCG1, CEL, TENM2, HAS2, SLC13A3, NGFR, SLC14A1, HS3ST3B1, KLRC1
10	GO:0005886~plasma membrane	CC	65	1.5	CYP24A1, CADM1, LYPD5, PREX1, UTRN, SUSD2, TNFSF15, KCNJ12, SPRY1, SLC16A1, CXCR4, GPC6, STRA6, KCNQ1, CEACAM1, HTR1F, EFR3B, CD3G, ACKR3, COLEC12, DLL1, WNK2, SIRPG, CPE,

					CA9, SERPINB2, CLDN2, MADCAM1, NGFR, KLRC1, WNT5A, SNAP91, CCR1, HMGCS1, FPR1, CDH1, BDKRB1, CLDN11, BDKRB2, PCSK9, CAMK2B, GPR20, BMF, IL2RB, VAV3, SLCO4C1, XKR3, NOX1, NTN4, DGKK, C4BPB, CAPN3, KCNK2, ABCG1, GGT5, OR2F2, SULF2, TENM2, RGS4, PECAM1, SLC13A3, RAB38, RGS9, SLC14A1, SYT17

Table 4.4: Significant KEGG pathway based on 216 differentially expressed genes.

Sr.	Term	Gene	Fold	Gene Name
1	hsa04610: Complement and coagulation cascades	5	6	SERPINA5, CFH, BDKRB1, C4BPB, BDKRB2
2	hsa04971: Gastric acid secretion	4	4.5	CAMK2B, PLCB1, KCNQ1, KCNK2
3	hsa04750: Inflammatory mediator regulation of TRP channels	5	4.2	CYP2J2, BDKRB1, CAMK2B, BDKRB2, PLCB1
4	hsa04725: Cholinergic synapse	5	3.7	CAMK2B, CREB3L3, KCNJ12, PLCB1, KCNQ1
5	hsa04670: Leukocyte transendothelial migration	5	3.6	VAV3, CXCR4, PECAM1, CLDN2, CLDN11

6	hsa04514: Cell adhesion molecules (CAMs)	6	3.5	CADM1, PECAM1, CLDN2, MADCAM1, CDH1, CLDN11
7	hsa04060: Cytokine-cytokine receptor interaction	9	3.1	INHBB, IL2RB, IL17A, CXCR4, CCR1, IL21R, TNFSF15, ACKR3, NGFR

4.2.3. PPI network of selected DEGs were created for identification of Hub genes

Protein-protein interaction (PPI) analysis through STRING database was performed using previously selected 216 GEGs where 216 nodes and 231 edges were observed. Next, obtained PPI network was analysed using Cytoscape, where we identified 139 hub genes (Fig. 3a). On the basis of degree centrality (i.e. the number of connections to other nodes) and betweenness centrality (i.e. lies on path linking other node pairs) hub genes(node) were ranked [26]. We observed that CDH1 hub gene has the highest degree centrality score with connections to 18 nodes (Fig. 3b). CDH1 also has highest betweenness centrality score. This shows that CDH1 has a central role in the interconnections of other node pairs in relation to TRAIL resistance in breast cancer.

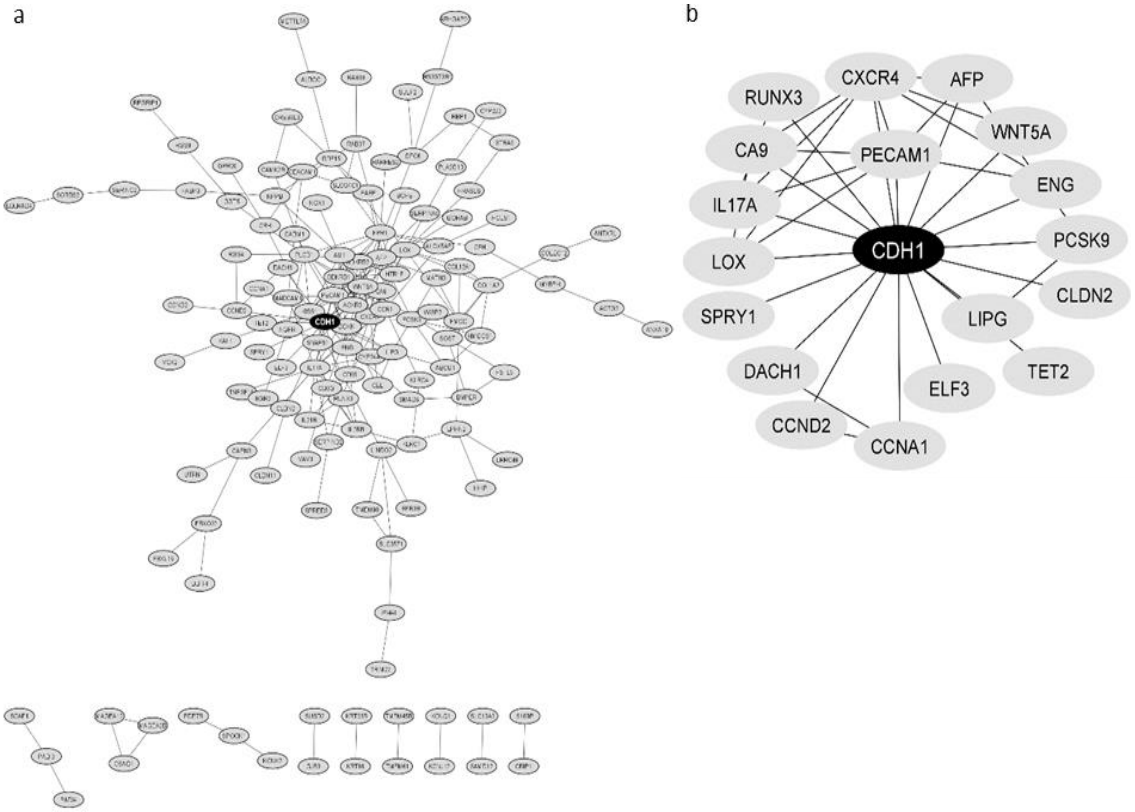

Figure 4.3. *PPI network analysis. (a) PPI network was constructed with 139 hub genes. (b) PPI network of CDH1 hub gene with 18-degree centrality, showing interaction of CDH1 with 18 different node genes. In both (a) and (b), CDH1 has been shown in black circle and the other hub genes have been shown in grey circle.*

4.2.4. Copy number variation at the CDH1 locus in rhTRAIL resistant cells might pay a role in decreased expression of CDH1 transcript

Microarray data was confirmed via quantitative real-time PCR showing downregulation of CDH1 transcript by 0.43-fold in TR cells with respect to TS cells ($P \leq 0.05$) (Fig. 4a). To understand the reason behind the decreased CDH1 mRNA expression level, CDH1 locus copy number variation (CNV) analysis was performed for TR cells and TS cells. copy number deletion was found at the CDH1 locus of TR cells when compared with TS cells (Fig. 4b). 953 TCGA breast cancer (BRCA) patient samples were analysed, there we found diploid CDH1 locus for 28.12% patients. Deletion and amplification were shown by the remaining 62.12% and 28.12% patients, respectively, at the CDH1 locus. Analysis of CDH1 gene expression level of TCGA BRCA patients showed a significant lower expression of CDH1 mRNA level in those patients who were having copy number deletion in comparison to patients with diploid copy of the CDH1 gene (Fig. 4c). This data indicates that decreased expression of the CDH1 transcript in the TR cells might be due to copy number deletion at CDH1 loci.

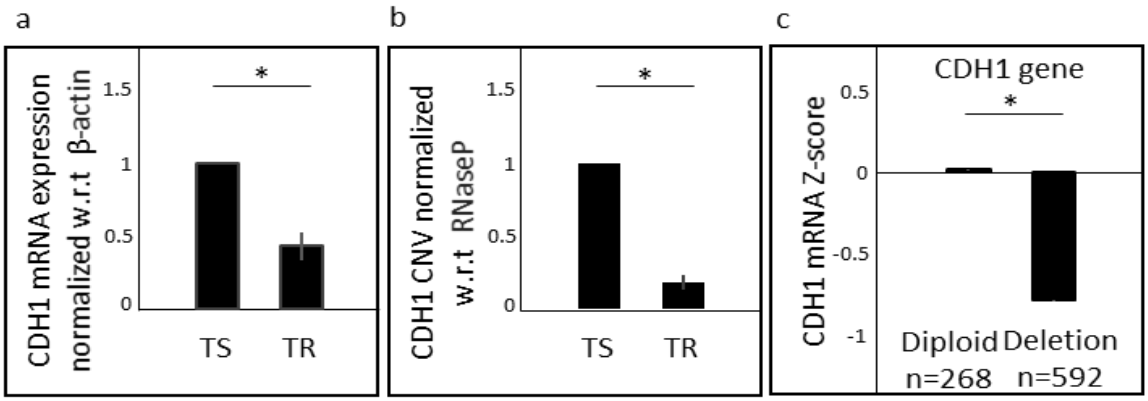

Figure 4.4. *MDA-MB-231 TR cells show reduced CDH1 expression. (a) Transcript analysis was carried out for TS and TR cells using real-time PCR. β-actin was used as an endogenous control. (b) Genomic DNA was analyzed to study copy number variation (CNV) at CDH1 locus in TS and TR cells. RNaseP was used as an endogenous control. Graphs shows average expression of three independent experiments normalized with*

respect to (w.r.t) the respective endogenous controls. (c) CNV analysis of CDH1 mRNA z-score in 953 TCGA patient samples.

4.2.5. Overexpression of CDH1 in TR cells showed increased apoptosis in comparison to TR parental cells

CDH1 western blot analysis showed lower abundance of the protein in TR cells in comparison to TS cells (Fig. 5a). For understanding if CDH1 plays any significant role in determining the rhTRAIL resistance nature MDA-MB-231 breast cancer cells, CDH1 gene and the respective control plasmid was overexpressed in TR and TS cells and confirmed in mRNA and protein level (Fig. 5b, 5c). Surprisingly, after overexpression of CDH1 we observed that cell death has significantly increased in transfected TR cells in comparison the parental cells and same trend was observed in case of decreased cell viability in transfected TR cells after treatment with rhTRAIL. ($P \leq 0.05$) (Fig. 5d, 5e). Furthermore, early and late apoptosis also showed notable increase percentage in CDH1 overexpressing, TR cells in comparison to the parental TR cells when treated with rhTRAIL (Fig. 5f, 5g). To confirm increase in apoptosis after overexpression of CDH1 in TRA cells, cleaved caspase-3 western blot was performed where we found increased expression of cleaved caspase-3 in TR cells that were overexpressing CDH1 in comparison to the parental TR cells expressing control plasmid after treatment with rhTRAIL (Fig. 5h). Therefore, we can conclude that CDH1 overexpression in TR cells sensitizes these cells towards rhTRAIL treatment.

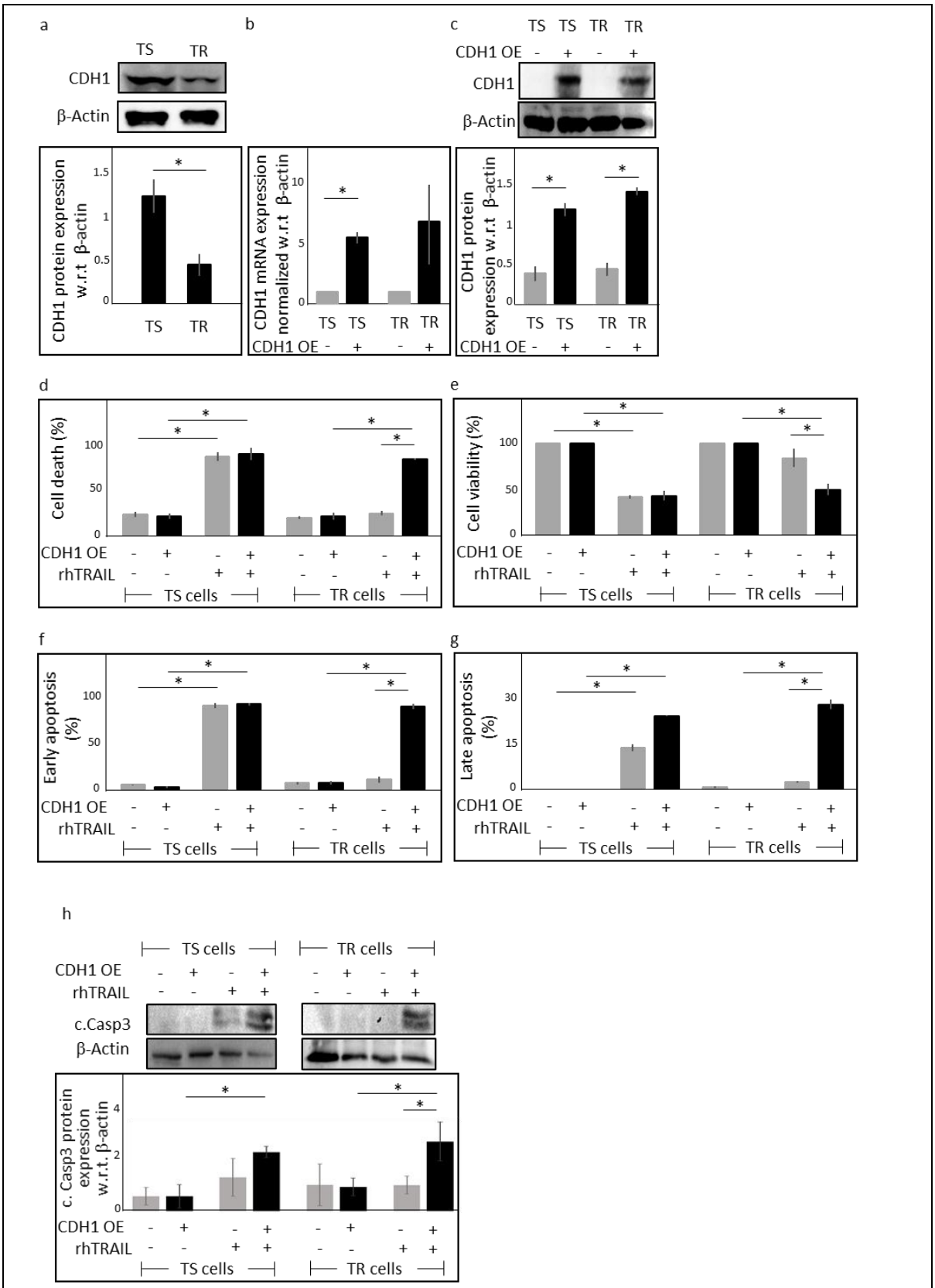

Figure 4.5. *Overexpression of CDH1 sensitizes TR cells towards TRAIL mediated toxicity. 48hrs posttransfection of CDH1 (or control plasmid) in TS and TR cells (a) CDH1 transcript was measured using realtime PCR and (b) CDH1 protein was measured using western blotting. β-actin was used as an endogenous control. Both quantitative PCR*

(qPCR) and immunoblot graphs shows average expression of two independent experiments normalized with respect to (w.r.t) endogenous control. (c) Trypan blue assay was carried out to measure cell death and (d) MTT assay was performed to measure cell viability in CDH1 (or control plasmid) overexpressing TS and TR cells after 24hrs of 500ng/ml rhTRAIL treatment. All experiments were carried out in triplicate and repeated three times. Representative experiment has been shown in the figure. (e) Early apoptosis and (f) late apoptosis was measured by AO/EtBr assay in CDH1 (or control plasmid) overexpressing TS and TR cells after 6hrs of 500ng/ml rhTRAIL treatment. All experiments were carried out in triplicate and repeated three times. Representative experiment has been shown in the figure. (g) Western hybridization showing cleaved caspase-3 in control plasmid and CDH1 transfected TS and TR cells after 6hrs of rhTRAIL (500ng/ml) treatment. β-actin was used as an endogenous control. Graph shows average expression of three independent experiments normalized with respect to (w.r.t) endogenous control.

4.2.6. CDH1 expression was found to be low in TRAIL resistant breast cancer patient samples in comparison to TRAIL sensitive patient samples

For better understanding of TRAIL resistant nature of cancerous cells, from published literature TRAIL sensitive and TRAIL resistant breast cancer cell lines were identified. (Table 4.5). Next, mRNA z-score values of TRAIL and its four receptors of these cell lines were used to calculate a numerical value, (TRAIL + DR4 + DR5)/ (TRAIL + DCR1 + DCR2), which we termed as the apoptosis/survival ratio (A/S ratio). Calculations showed that A/S ratio value of TRAIL sensitive cell line were higher in comparison to the A/S ratio value of TRAIL resistant cell lines. (Fig. 6a). Thereby, we can conclude that the A/S ratio obtained from the cell line study can predict the resistant or sensitive nature of cancerous cells against TRAIL treatment. Then we divided the 953 breast cancer patient samples which were obtained from TCGA database as per there A/S ratio, categorising them into TRAIL resistant (low A/S ratio) group and TRAIL sensitive (high A/S ratio) group. Interestingly, we observed that the TRAIL sensitive group showed significantly increased CDH1 mRNA expression in comparison to the TRAIL resistant group (Fig. 6b). When we further segregated these patient samples as per there stages, there also we found that CDH1 transcript expression to be significantly higher in the TRAIL sensitive group in comparison to the TRAIL resistant group in all the three stages (Fig. 6c). This shows that high CDH1 mRNA expression is consistent throughout stage I, II and III breast cancer patients in TRAIL sensitive group irrespective of tumor stage.

Table 4.5: TRAIL resistant and sensitive breast cancer cell lines

Sr.	Cell line	TRAIL
1	BT549	Sensitive[23,163]
2	MDAMB231	Sensitive[23,163,164]
3	HCC38	Sensitive[23,163]
4	CAMA1	Sensitive[165]
5	HS578T	Sensitive[23,163]
6	SKBR3	Resistant[23,163,164]
7	HCC1937	Resistant[23,163,164]
8	HCC1395	Resistant[23]
9	BT474	Resistant[23,163,164]
10	T47D	Resistant[23,163,164]
11	MCF7	Resistant[23,163,164]
12	MDAMB361	Resistant[164]
13	MDAMB453	Resistant[23,163]
14	HCC1500	Resistant[23,163]
15	AU565	Resistant[23,163]
16	ZR751	Resistant[23,163,164]
17	HCC1954	Resistant[23,163]
18	HCC1599	Resistant[23]
19	HCC1187	Resistant[23]

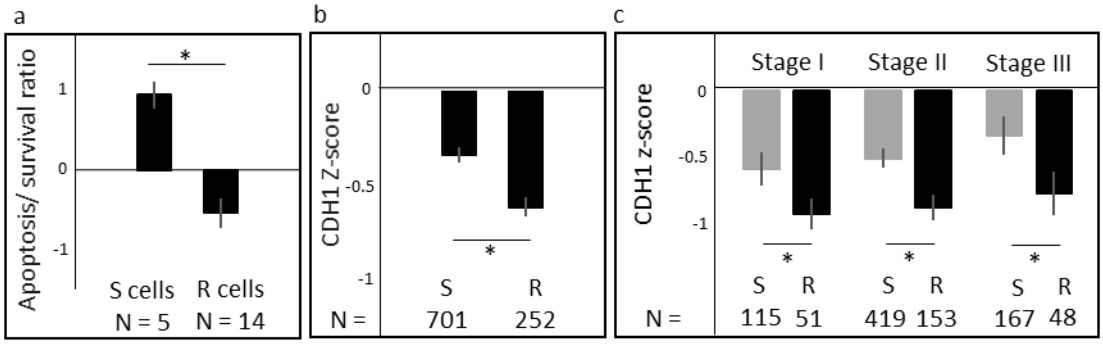

Figure 4.6. *TCGA breast cancer patient group with low A/S ratio shows reduced expression of CDH1 mRNA level. (a) Apoptosis/ survival (A/S) ratio using mRNA z-score of (TRAIL+DR4+DR5)/ (TRAIL+DCR1+DCR2) of TRAIL-sensitive and -resistant cell lines. (b) CDH1 mRNA expression of TRAIL-sensitive and -resistant patient groups. (c) CDH1 z-score of breast cancer tumor stage I, II, and III of the TRAIL-sensitive and -resistant patient groups.*

4.2.7. CDH1 regulates the DR4 in TRAIL resistant cells

In order to identify the mechanism underling CDH1 sensitizing TRAIL resistant cells towards apoptosis, death receptors were analysed. Surprisingly Overexpression of CDH1

have increased the expression of DR4 in resistant cells after overexpression of CDH1 in TR cells and rhTRAIL treatment does not have any effect on DR4 expression in presence or absence of CDH1 in TS or TR cells (Fig. 7a). We observed CDH1 (Pearson's correlation coefficient, r = 0.13, p < 0.05) to be positively correlated with DR4 in TCGA breast cancer patient samples (Fig. 7b)

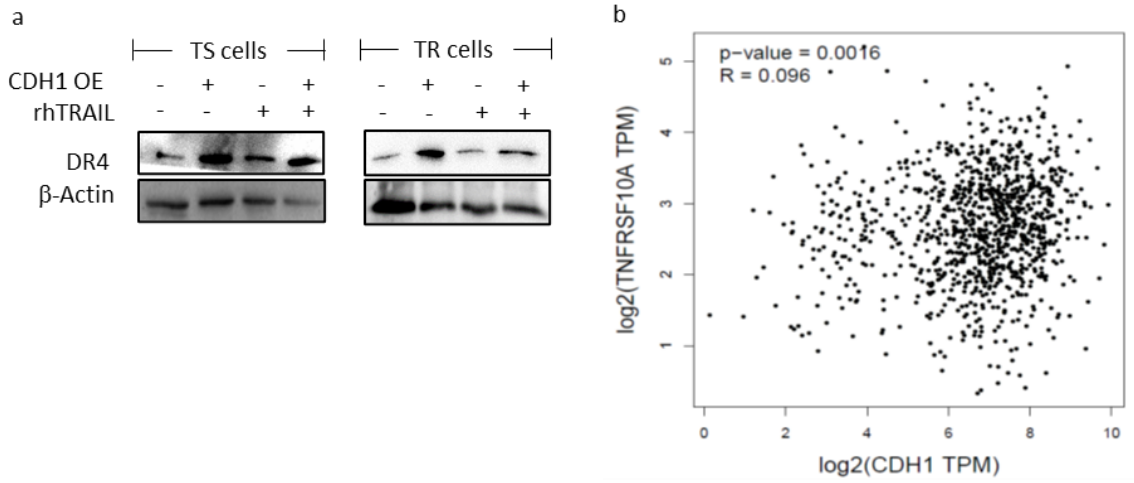

Figure 4.7. *CDH1 increases DR4 in TR cells. (a) Western blot hybridization showing DR4 expression in control plasmid and CDH1 transfected TS and TR cells after 6hrs of rhTRAIL (500ng/ml) treatment. β-actin was used as an endogenous control. (b)Positive correlation was found between CDH1 and DR4 transcript expression in the TCGA breast cancer patient samples (r = 0.096, p <0.05)(TPM=Transcript Per Million).*

4.2.8. Discussion

In the current study we are trying to understand the mechanism underlining the TRAIL-resistance, because TRAIL-receptors are expressed selectively only on the tumour cell surface, therefore it acts on the tumor cells without affecting normal cells. The detailed mechanisms of TRAIL-resistance are not well understood. So far, many studies have reported that the main mechanism is down-regulation of death receptors, which is common to various cancer cell types[63]. Via whole transcriptome analysis of the TRAIL resistant cells we have attempted to understand the molecular mechanisms underlying resistance offered against TRAIL induced cell death. In this study, we clarified that the mechanism of TRAIL-resistance consisted of downregulation of CDH1, as evident from the results obtained by using inherent TRAIL-resistant triple negative breast cancer MDA-MB-231 cells and sensitive MDA-MB-231 cells.

For our study, MDA-MB-231 breast cancer cells were selected as they are known to be sensitive to TRAIL induced cell death. However, eliminating cancer cells have always been a challenge because of their highly heterogeneous in nature. It has been already shown that MDA-MB-231 cells consists of mixed population of cells. We observed that on treating the TRAIL sensitive population of MDA-MB-231 cells with very high dose of 800ng/ml rhTRAIL (IC99) we were able to select a subpopulation of cells that were inherently resistant to TRAIL mediated cell death. For the purpose of downstream experiments, the parental population of TRAIL sensitive (TS) cells and the selected subpopulation of TRAIL resistant (TR) cells were exposed to 500 ng/ml of rhTRAIL followed by performing assays to analyze cell viability, cell death and apoptosis. We observed that the TR cells showed reduced cell death and increased cell viability on rhTRAIL treatment in comparison to control TS cells that had been exposed to rhTRAIL (Fig. 1a, 1b). We further observed that subpopulation of TR cells that were obtained after exposure to 800ng/ml of rhTRAIL showed reduced apoptosis on treatment with 500ng/ml rhTRAIL in comparison to TS cells that had been exposed to 500ng/ml rhTRAIL (Fig. 1c, 1d, 1e, 1f). The above experiments confirmed that the TR cells that we have generated were indeed resistant to rhTRAIL mediated apoptosis.

Microarray studies have immense potential to provide unique understanding into the pathogenesis of complex disease. Through whole transcriptome analysis of TR MDA-MB-231 cells we identified 216 transcripts having mRNA accession number and fold change of greater than +2 or less than -2 that were differentially expressed with respect to the TS cells. Out of these 216 genes, 83 genes were upregulated and 133 genes were downregulated in the TR cells (Fig. 2a, 2b, Table 4.1, 4.2). In order to investigate the interactions among these DEGs, GO and KEGG pathway analyses was carried out through DAVID. The most significant biological process ‘response to organic substance’ obtained by GO pathway analysis of 216 DEGs showed a 16.6-fold enrichment (Fig. 2c, 2d, Table 4.3, 4.4). Response to organic substance’ comprised of WNT5A, CRIP1, S100P, CDH1, ABCG1. Next, PPI network analysis was carried out to identify the hub genes related to endogenous TRAIL resistance in breast cancer. We found CDH1 to be the gene with the highest degree centrality and maximum betweenness centrality indicating that it one of the most important genes among the 216 DEGs (Fig. 3a, 3b). Lu et.al. showed CDH1 to regulate TRAIL mediated apoptosis via its interaction with actin cytoskeleton. Moreover, low expression of CDH1 has been correlated with less chances of survival in breast cancer

patients. Therefore, CDH1 was chosen for further evaluation in the context of TRAIL resistance in MDA-MB-231 breast cancer cells.

Quantitative real-time PCR (qPCR) is a frequently used to validate gene expression results obtained from microarray analysis. Therefore, we confirmed the downregulation of CDH1 transcript in TR cells using real-time PCR (Fig. 4a). Downregulation of tumor suppressor CDH1 gene has been reported in many cancers. The most common allelic alterations in breast cancer were found to be the loss of an allele at the CDH1 locus (16q22.1). Therefore, by performing CNV analysis we wanted to examine if the loss of this tumor suppressor gene at the genomic level was playing any role in its decreased expression at the transcript level. qPCR analysis using genomic DNA (gDNA) showed copy number deletion at the CDH1 gene locus of TR cells compared to TS cells (Fig. 4b). Moreover, analysis of the 953 TCGA breast cancer samples showed CDH1 copy number loss to be responsible for reduced CDH1 transcript expression in comparison to the patients with diploid copy of the CDH1 gene (Fig. 4c). The data suggests downregulation of CDH1 gene in the rhTRAIL resistant cells could be due to the loss in copy of the CDH1 gene.

We further observed CDH1 protein to be downregulated in the TR cells in comparison to the parental TS cells (Fig. 5a). In order to understand the role of CDH1 in regulating the response of breast cancer cells to TRAIL mediated cell death CDH1 gene was overexpressed in TR and TS cells (Fig. 5b, 5c). After overexpression of CDH1 gene in TR cells, we observed increased cell death and reduced cell viability after treatment with rhTRAIL treatment in comparison to TR cells transfected with vector control that had been treated with similar amount of rhTRAIL. However, overexpressing CDH1 in TS cells did not have any additional effect with respect to rhTRAIL sensitivity (Fig. 5d, 5e). Furthermore, we observed that overexpressing CDH1 in TR cells was capable of enhancing both early as well as late apoptosis in these cells after rhTRAIL treatment in comparison to rhTRAIL treated vector transfected TR (Fig. 5f, 5g). Since active caspase-3 is the main determinant of apoptos, we wanted to study the effect of CDH1 overexpression on rhTRAIL mediated caspase-3 activation in the TR and TS cells. After overexpression of CDH1 in TR cells, we even observed increased level of cleaved caspase-3 on treatment with rhTRAIL in comparison to the control vector expressing TR cells that had been treated with rhTRAIL (Fig. 5h). Thus, from the above experiments we could conclude that lower expression level of CDH1 gene in inherent TRAIL resistant cells plays an important role in regulating sensitivity towards rhTRAIL mediated apoptosis. However,

the resistant nature of these cells can be reverted back towards their original sensitive nature via upregulation of CDH1 in these TRAIL resistant MDA-MB-231 breast cancer cells. The reason behind the sensitization of these resistant cells towards TRAIL induced apoptosis could be because of increased DR4 expression level in CDH1 overexpressing TR cells (Fig. 7a). Interestingly we also found positive correlation between DR4 and CDH1 in TCGA breast cancer patient sample (Fig. 7b).

Breast cancer cell lines show different grades of response towards TRAIL mediated cytotoxicity. Based on extensive literature survey, we identified 5 TRAIL sensitive and 14 TRAIL resistant breast cancer cell lines. mRNA z-score values of TRAIL and its four receptors that was obtained from cBioportal was categorized into pro-apoptotic versus pro-survival signal at the level of TRAIL and its receptors. We observed that the apoptosis/ survival (A/S) ratio is natablely high in TRAIL sensitive cells in comparison to the TRAIL resistant cells, indicating that sensitivity of the cells towards TRAIL treatment can be predicted via A/S ratio. (Fig. 6a). On categorizing the 953 TCGA breast cancer patients based on the abovementioned A/S ratio we found that the patient group with high A/S ratio (TRAIL sensitive) showed higher expression of CDH1 gene mRNA level in comparison to the group with low A/S ratio (TRAIL resistant) (Fig. 6b). Thereafter, further subdivision of the 953 breast tumor patients on the basis of tumor stages, further showed that stage I, II and III tumors exhibited significantly high CDH1 transcript in the the TRAIL sensitive group with respect to TRAIL resistant group of patients (Fig. 6c). However, this difference was not observed in stage IV tumors (data not shown). Therefore, from the above analysis we can infer that expression level of CDH1 can be a determining factor for TRAIL resistance in breast cancer.

Taken together, we can conclude that MDA-MB-231 breast cancer cells are heterogeneous for response to rhTRAIL treatment. These TRAIL sensitive cells consist of a subpopulation of cells that have deletion in a copy of CDH1 gene. This causes subsequent reduction in the transcript as well as protein expression of CDH1 gene, making them resistant to rhTRAIL mediated apoptosis. Overexpression of CDH1 in these rhTRAIL resistant cells results in increased expression of DR4 thereby making these cells sensitive to rhTRAIL mediated apoptosis (Fig. 8). Thus, our study suggests CDH1 can be utilized as a biomarker for personalised TRAIL therapy for the patients, as we observed low expression of CDH1 in TRAIL resistant breast cancer patient cohort.

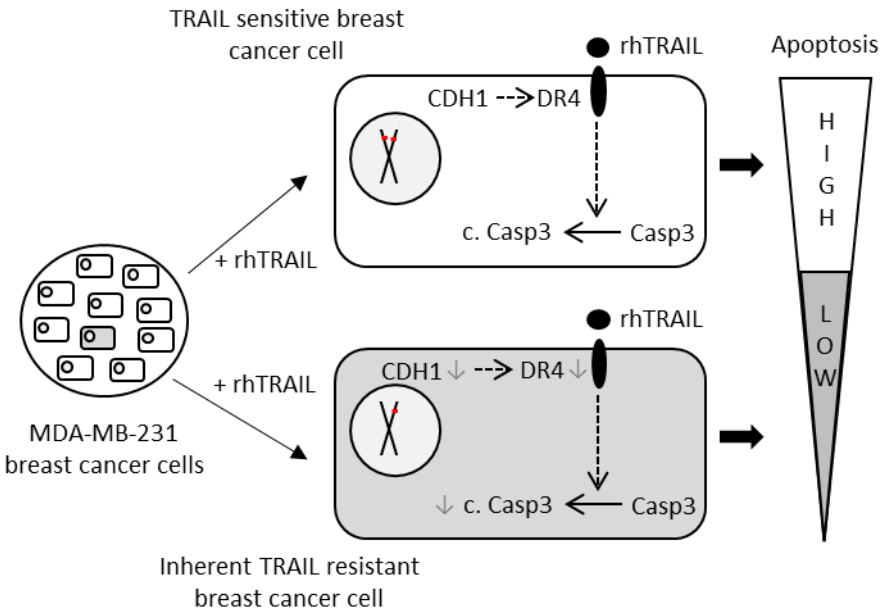

Figure 4.8. *Graphical representation of the proposed mechanism of TRAIL sensitivity in MDA-MB-231 breast cancer cells. TRAIL sensitive MDA-MB-231 breast cancer cells consist of a sub-population of cells that are resistant to TRAIL mediated cell death. These inherent TRAIL resistant cells show deletion of the CDH1 locus (black box on the chromosome) thereby resulting in reduced CDH1 expression. Decreased expression of CDH1 causes reduction in the expression TRAIL receptor, DR4, by an unknown mechanism thus rendering them less susceptible to rhTRAIL induced apoptosis. Normal expression of CDH1 and subsequently, DR4, in control MDA-MB-231 cells results in increased apoptosis after rhTRAIL treatment. (N: nucleus; C: Cytosol).*