Glioblastoma

Kelly Kozakiewicz

Glioblastoma can be categorized into four subtypes. These four subtypes are based off of gene expression-based molecular classification and are known as Proneural, Neural, Classical and Mesenchymal. To characterize these subtypes they are based off of the abnormalities of PDGFRA, IDH1, EGFR, and NF1. This paper will begin by discussing what glioblastoma is, the type of neurons this tumor intends to attack, looking at the two subtypes of proneural and classical, along with the two abnormalities associated with those subtypes being EGFR and IDH1. The research paper will then begin to look at the relevance of EGFR and IDH1 on inhibition or over expression of genes, and then take a look at microRNAs associated with glioblastoma.

Glioblastoma is a brain tumor and is also known as a grade IV astrocytoma. The astrocytoma relates to the specific neurons known as astrocytes. Astrocytes are the most abundant neurons of the brain, and perform many functions such as biochemical support of endothelial cells which forms the blood brain barrier. However, this type of brain tumor can have a mixture of all types of neurons. It does not pin point to just one type of neuron. There are no known causes of how this type of cancer arises, but research is still being conducted today in order to figure that question out. This type of cancer is the most malignant of its kind, and according to the American Brain Tumor Association, the reason glioblastoma can be distinguished is by its histological features. They state in their brochure, "The histologic features that distinguish glioblastoma from all other grades are the presences of necrosis (dead cells) and increase of blood vessels around the tumor." This gives some researchers insight into the histological features, but still not enough is known about the pathophysiology of how the cancer arises in the tissue.

Glioblastoma has been put into four subtypes. The main focus in this paper will be on the proneural and classical subtypes. The proneural subtype has two main features that accompany it. These two features would be the alteration of the PDGFRA and point mutations that occur in the isocitrate dehydrogenase 1 (IDH1) (Verhaak et al). The main focus in the proneural subtype will be on the IDH1. The next subtype is classical, which is where chromosome 7 amplification is paired with chromosome 10 loss. This is highly frequent in glioblastoma, and where there is high-level of epidermal growth factor receptor (EGRF) amplification (Verhaak et al). In the classical subtype the emphasis will be on the EGRF. Table 1, below, shows the copy number alterations that correlate with the gliobastoma subtypes.

Type of Event, ROI	Proneural (n = 54)	Neural (n = 24)	Classical (n = 37)	Mesenchymal (n = 55)	Total No. of Samples Altered	Known Cancer Gene in Region
Low- and High-Level Amplified Events						
7p11.2	**29 (54%)**[a]	23 (96%)	37 (100%)	52 (95%)	141	*EGFR*
7q21.2	**25 (46%)**[a]	23 (96%)	34 (92%)	49 (89%)	131	*CDK6*
7q31.2	**29 (54%)**[a]	22 (92%)	32 (86%)	50 (91%)	133	*MET*
7q34	**28 (52%)**[a]	22 (92%)	32 (86%)	50 (91%)	132	
High Level Amplification Events						
7p11.2	**9 (17%)**[a]	16 (67%)	**35 (95%)**[a]	16 (29%)	76	*EGFR*
4q12	**19 (35%)**[a]	3 (13%)	2 (5%)	5 (9%)	29	*PDGFRA*[b]
Homozygous and Hemizygous Deletion Events						
17q11.2	3 (6%)	4 (17%)	2 (5%)	**21 (38%)**[a]	30	*NF1*
10q23	**37 (69%)**	23 (96%)	37 (100%)	48 (87%)	145	*PTEN*
9p21.3	30 (56%)	17 (71%)	**35 (95%)**	37 (67%)	119	*CDKN2A/CDKN2B*
13q14	28 (52%)	11 (46%)	**6 (16%)**	29 (53%)	74	*RB1*
Homozygous Deletion Events						
9p21.3	22 (41%)	13 (54%)	**34 (92%)**[a]	29 (53%)	98	*CDKN2A/CDKN2B*

ROI, region of interest. Significance of the difference in number of events between subtypes and remainder of the subtypes was tested using a two-sided Fisher's exact test, corrected for multiple testing using a Familywise Error Rate. Bold type indicates p values significant at 0.1 level. Also see Figure S4 and Table S1.

[a] p value significant at 0.01 level.

[b] The peak of the amplification is adjacent to *PDGFRA*.

Table 1

Epidermal Growth Factor Receptor

In this experiment performed by Romeike et al, they took 23 micro dissected areas of 10 glioblastomas to investigate for quantitative genomic aberrations using comparative genomic hybridization (CGH). After the micro dissection, they took parallel tissue sections that were stained immunohistochemically with an antibody that detects both wild-type EGFR and deletion mutant form of the receptor EGFRvIII. Chromosome 7 is usually looked at with the comparative genomic hybridization because it is the most frequent aberrant chromosome in glioblastoma. The EGRF gene is involved in cell proliferation control and is located on chromosome 7 and encodes a transmembrane receptor protein with binding specificity for EGRF and transforming growth factor alpha. This gene is highly overexpressed and sometimes shortened and in a rearranged form. They correlated findings of previous studies in order to see the concern of chromosome 7 with immunohistochemistry of EGFR (Romeike et al).

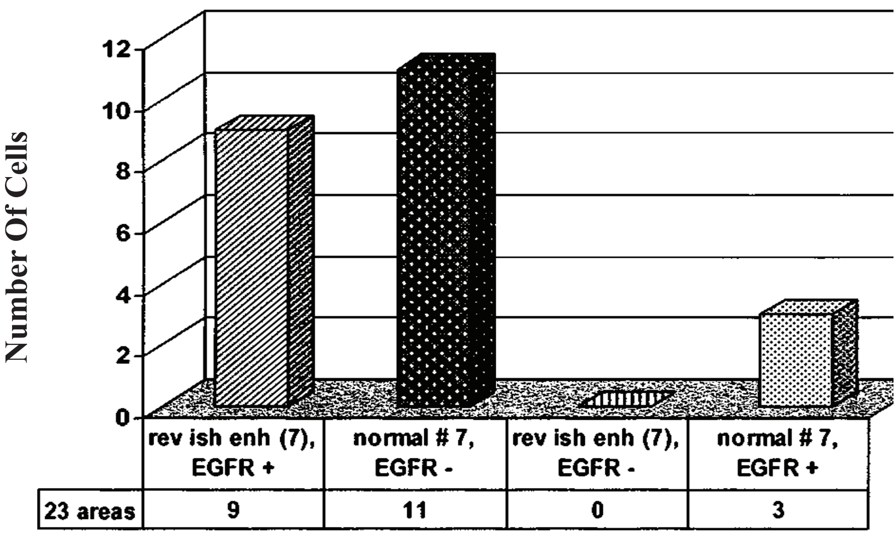

Fig. 1. Comparison of the chromosomal findings of chromosome 7 as revealed by CGH after micro dissection with the detection of EGFR-immunoreactivity.

For the CGH testing, they took 23 areas from 10 glioblastoma to microdissect from a 10 micrometer thick formalin-fixed, paraffin-embedded tissue sections. In this the whole DNA was amplified evenly with DOP-PCR and screened for genetic imbalances. For the immunohistochemistry staining, they took parallel tissue sections of the micro dissected sections using avidinbiotin peroxidase method. They used a monoclonal mouse anti-human EGFR antibody that recognizes 170kD wild-type and the 145 kD deletion mutant form of the receptor. The antibody was applied at a dilution of 1:200 for an incubation period of 1 hour at 37C. To have a control tissue, they used stratified squamous epithelium, with a negative control by staining stratified squamous epithelium with substitution of the primary antibody with antibody diluent. By doing this, they could stain normal adult brain tissue, which showed no EGRF immunoreactivity.

Table 2, below, shows the comparison of chromosomal irregularities of CGH after microdessection and EGFR immunohistochemistry in 10 glioblastomas.

Tumor No	age/ sex[a]	area	CGH karyotype of microdissected probe[b]	EGFR immuno-histochemistry
N235/93 primary GBM[c]	58/f	1.1 epithelial	rev ish enh (5q13q23, 6q15q22, 12q21q22, 13q21) dim (22)	–
		1.2 diffusely grown	rev ish enh (4, 5q13q23, 6q15q22, 7, 8, 12q21q22, 13q21, X) dim (22)	+
N416/94 primary GBM[c]	52/m	2.1 diffusely isomorphic	rev ish enh (4q31, 5q14q33, 6q, 12q15, 13q21q31)	–
		2.2 diffusely pleomorphic	rev ish enh (4q21q31, 5q14q33, 6q, 8q13q23, 12q15, 13q21q31)	–
		2.3 densely grown	rev ish enh (4q21q26)	–
recurrency: N1477/94	52/m	3.1 diffusely grown	rev ish enh (1p31p33, 2q21q31, 4q13q31, 5q14q22, 7, 8, 12q21, 13q13q22)	+
		3.2 densely grown	46,XY	–
N828/94 primary GBM[c]	59/m	4.1 densely grown	rev ish enh (4q11q31, 12q15q22, 13q14q22) dim(22)	-
		4.2 diffusely grown	rev ish enh (12q15q22)	+
N1254/94 primary GBM[c]	31/m	5.1 epithelial	rev ish enh (20q11q12)	–
N215/95 primary GBM[c]	64/m	6.1 densely grown	rev ish enh (4q24q28, 13q21) dim (1p34pter, 10q26qter, 11p15pter, 17p, 19q13qter, 22)	–
		6.2 diffusely grown	rev ish enh (5q12q31, 12q21) dim (1p36pter, 22)	–
N790/96 primary GBM[c]	79/m	7.1 diffusely grown	rev ish dim (1p36pter, 10q26qter, 19, 22q12qter)	–
N1194/96 primary GBM[c]	56/f	8.1 gemistocytic	rev ish enh (11q25)	+
		8.2 densely grown	rev ish enh (4q34qter, 19q13qter, 22)	–
N1242/96 primary GBM[c]	73/f	9.1 densely grown	rev ish dim (22)	+
		9.2 diffusely grown	rev ish enh (7, X) dim (22)	+
		9.3 gemistocytic	rev ish enh (7) dim (19p) amp (7p21)	+
N190/97 primary GBM[c]	66/m	10.1 subpial grouping	rev ish enh (4q13q21, 7, 12q13q15, 20p13) dim (17p11)	+
		10.2 satellitosis	rev ish enh (1p33p34, 7, 17p11p12, 20p12pter) dim (9p13pter) amp (7p11.2)	+
		10.3 five giant nuclei	rev ish enh (7, 20p12pter) dim (Y)	+
		10.4 pseudopalisading	rev ish enh (1p13pter, 6p21pter, 7, 19, 20, 22q12qter) dim (Y)	+
		10.5 diffusely pleomorphic	rev ish enh (7, 16p21, 19) dim (Y)	+

[a] m, male; f, female
[b] The description of karyotypes is based on the ISCN 1995 (8): rev ish, reverse *in situ* hybridization; enh, enhanced fluorescence ratio intensity - means gain in one copy of a given region; dim, diminished fluorescence ratio intensity - means loss of one copy of a given region; amp, amplification.
[c] GBM, Glioblastoma multiforme

Table 2

From this study, it showed that nine of 23 areas with tumor tissue from 10 glioblastomas showed a gain in chromosome 7, and two of these an additional overexpression on 7p with CGH after microdissection.

This is shown from the figure above. These areas all contained EGFR-immunoreactive cells on the parallel tissue sections. Since this gene is localized on chromosome 7, there is around 40% amplified EGRF genes in glioblastoma and 50% with genomic rearrangement. Only three out of 14 areas showed no abnormalities of chromosome 7 containing EGFR-immunopositive cells.

In the study done by Ayuso et al, they hypothesized that the expression and mutation in post-natal neural stem cells may contribute to cellular proliferation, survival and migration. They believe that this would give many people better understandings and insights to understanding the neural stem cells which could help down the road with finding deregulated mechanisms for glioblastoma tumor stem cell invasion, proliferation, survival and resistance to the current treatment options of patients.

For this experiment, human glioblastoma samples were collected for comparison of baseline wild type EGFR and EGFRvIII expression levels. Fresh tumor tissue was taken from patients with resection of human of glioblastoma and then they used mouse C17.2 neural stem cells that were grown in M2 media and differentiation media. They then used a variety of methods to test for the expression of EGFR by the following; vector construction, RT-PCR, oxidative stress measurement, immunocytochemistry, western blot, MRR viability assay, cell cycle analysis, apoptosis assay, transmigration assay, and differentiation assay. The focal point of the methods will be on RT-PCR, western blot, and apoptosis assay.

In the RT-PCR the main focus was to see if and where the presence of wild type EGFR or EGFRvIII was in the mouse C17.2 subclones. From this they found that there is both in the C17.2 subclones. Figure 2, below, shows where they are located.

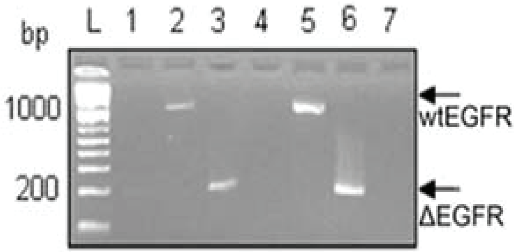

Figure 2 Presence of wtEGFR and EGFRvIII in C17.2

The western blot test was also used to see if the wild type EGFR and EGFRvII was detected in the neural stem cells in C17.2 wild type EGFR and the EGFRvIII clones. There was no significant amount of protein found in the C17.2 parental neural stems cells or in the neural stem cells that were infected with empty vector controls. In order to test the over-expression of the EGFRvIII or wtEGFR to induce the activation of downstream neural stem cells, the western blow was used with the presence or absence of the EGF ligand. From this they found that there was a constant up regulation of pPLCγ found in the wild type EGFR with or without the presence of EGF ligand. The activated EGFR signals in immortalized neural stems cells was associated with the activation of those downstream mediators widely implicated in glioblastoma tumor progression. They found that cell signaling pathways commonly implicated in glioma progression are important in postnatal neural stem cells that have activated EGFR signals. Below shows a figure with C17.2 cells that were cultured, serum starved and the addition of EGF.

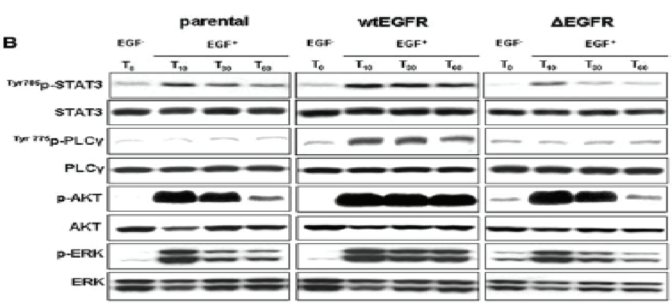

Figure 3: Activation of EGFR downstream pathways in C17.2 cells that over-express EGFR and EGFRvIII.

Apoptosis assay is the next key test we will be looking at. This was done using a total of 10,000 cells per sample and was repeated three times. From this they wanted to assess whether the increase in cell number of C17.2EGFRvIII and wtEGFR neural stem cells, in addition to proliferation, were a consequence of survival from the EGFR activation. The neural stem cells were cultured and stained with annexin V and PI. The serum-containing media no significant differences were found between the groups. In the serum-free group, the C17.2 parental cells early or late apoptosis. However, wtEGFR over-expression increased the neural stem cell survival entered, and the EGFRvIII over expression also increased survival. Adding ligand did not affect the surviving cells, but the addition of AG1478 highly increased the number of apoptotic cells in the subclones. This suggests that activated EGFR signaling improves survival and prevents apoptosis of post-natal neural stem cells. (Ayuso et al).

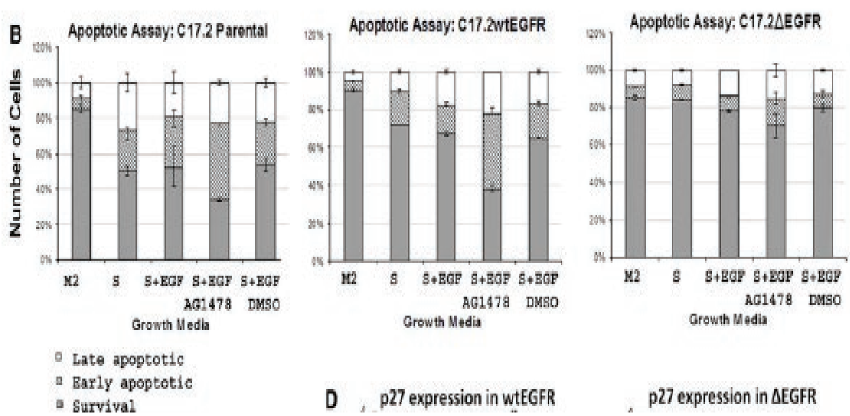

Figure 4 Apoptosis Assay

In this test, they also checked the expression of proapoptosis markers Bax and Bad. In the serum free media, an increase of Bax and Bad gene expression in the parental C17.2 neural stem cells compared to the C17.2wtEGFR or EGFRvIII neural stem cells. When they were grown in media with the presence of the EGFR inhibitor 5 μM AG1478, the Bad gene expression levels highly increased, which could suggest the increase survival of neural stem cells over expressing EGFR or EGFRvIII.

Isocitrate Dehydrogenase 1 Gene

The isocitrate Dehydrogenase 1 gene is located on the chromosome region 2q33 and belongs to a group of five genes that encode human isocitrate dehydrogenases. These are enzymes that catalyze the reaction of transition of isocitrate to alpha-ketoglutarate. IDH1 is the only one that is not mitochondrial and is present mainly in the cytoplasm and peroxisomes. They are still unclear on the direct job that IDH1, but they presume it plays a crucial role in cytoplasmic NADPH as well as fatty acid oxidation and degradation of phytanic acid in peroxisomes (Bujko et al).

In the study done by Bujko et al, they extracted DNA from a formalin-fixed, paraffin-embedded tissue. To determine mutation status, they used 58 patients, and used the PCR-RFLP method to screen for IDH1 codon 132 mutation. The hot spot for these mutations are located on exon 4, codon 132, which is where it encodes arginine on an active site of that enzyme. Bujko et al, developed a restriction fragment length polymorphism (RFLP) method to detect the frequent IDH1 mutations, R132H and R132C.

They used 58 patients who were newly diagnosed with glioblastoma that were treated with standard radiotherapy with temozolomide and performed a survival analysis wild-type vs. mutated patients. The DNA samples were obtained using the Scherlock AX kit, and quality of these samples were determined from a multiplex PCR reaction with a set of primers that amplify PCR products. The first step in the PCR was using 166bp fragment of IDH1 exon 4 to be amplified. It had to be diluted ten times and reamplified in two parallel reaction with two sets of primers designed for R132H and R132C mutation discrimination. The second primer used was a mismatch to create a control restriction site for the same enzyme that was used for mutation detection. These results had a false negative due to incomplete digestion by the specific enzyme used.

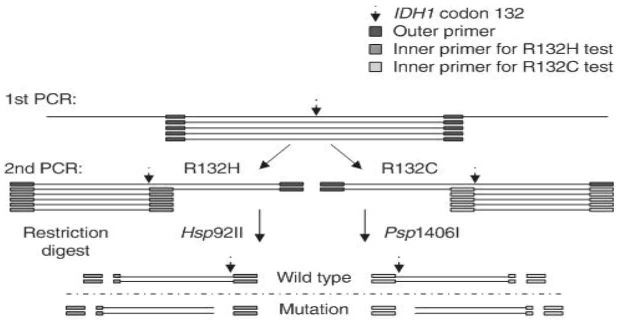

Fig. 1. PCR-RFLP procedure scheme.

Figure 3 PCR-RFLP procedure scheme

Above is a procedure on how the PCR was set up for this experiment. In this experiment, 14% of patients had the IDH1 R132H mutations. With the approach they did, they were able to look at these point mutations and look at the prognostic factors. IDH1 has significant prognostics in glioblastoma when not associated with temozolomide treatment or chemotherapy (Mellai et al). These mutations occurred more in younger patients, and correlated with a better progression-free survival over the survival of patients just treated with normal radiotherapy and temozolomide.

Researchers now are beginning to look into microRNAs, which are short noncoding ribonucleic acid molecules, that are approximately 22-nucleotide long and single stranded. MicroRNAs are post-transcriptional regulators that bind to complementary sequences on target mRNA, resulting in translational repression and gene silencing. This allows to modulate a variety of biological processes including, but not limited to, cell growth, proliferation, differentiation, metabolism and apoptosis (Wang et al).

Some studies have shown some microRNAs that are significantly altered in glioblastoma. One microRNA that is being studied is miR-221 because it has a preeminent position in the development of glioblastoma. It is a likely candidate for the role of tumor specific marker in glioblastoma (Ciafrè et al). In another study done by Silber et al, they took a look at miR-124 and miR-137, both of which have promising therapeutic value to patients with the glioblastoma disease. These microRNAs are expressed at lower levels, are up-regulated during neuronal differentiation of adult mouse neural stem cells induced by growth factor withdrawal, promote neural-like differentiation of growth-factor deprived mouse neural stem cells, mouse oligodendroglioma tumor stem cells, and human glioblastoma multiforme derived stem cells cell cycle arrest. These findings could allow for research to target the delivery of these microRNAs for better treatment options.

Glioblastoma multiforme and anaplastic astrocytoma				Glioblastoma multiforme only			Anaplastic astrocytoma only		
	Fold change glioblastoma multiforme	Fold change anaplastic astrocytoma	Ref		Fold change glioblastoma multiforme	Ref		Fold change anaplastic astrocytoma	Ref
Down-regulated				Down-regulated			Down-regulated		
miR-7	34	13		miR-101	4		miR-125b	153	[22]
miR-31	17	9		miR-128a	27	[22]	miR-126	7	
miR-107	14	10		miR-132	7		miR-127	149	
miR-124	57	26		**miR-133a**	17		miR-134	9	
miR-124b	45	27		**miR-133b**	14				
miR-129	43	27		miR-149	9		Up-regulated		
miR-137	63	66		**miR-153**	12		miR-296	11	
miR-138	27	12	[21]	miR-154*	7		miR-320	5	
miR-139	66	34		miR-185	5				
miR-187	6	5		miR-29b	6				
miR-203	13	4		miR-323	17				
miR-218	40	16		miR-328	8				
				miR-330	18				
Up-regulated									
miR-10b	35	75	[22]	Up-regulated					
				miR-21	7	[21,22]			
				miR-155	8				
				miR-210	7				

P-values for all miRNAs are $P < 0.05$ for comparisons of miRNA expression in tumors versus glioses; for miRNAs in bold $P < 0.01$. References indicate high-grade astrocytoma miRNA expression studies in which miRNA has been described previously.

Table 3 Differentially expressed microRNAs in anaplastic astrocytoma and/or glioblastoma multiforme tumors relative to non-neoplastic brain tissue.

The last microRNA that we will be looking at is MiR-18a because the expression of this microRNA was highly increased in glioblastoma tissues and cell line, while neogenin was down regulated. These are directly inverse of each other. These both correlate to the pathological grade of glimoa. MiR-18a in this case negatively regulates the mRNA and protein expression of neogenin by binding to a specific site within the neogenin. If miR-18a could be inhibited, it could potentially change the human glioblastoma phenotype by inhibiting cellular proliferation, migration and invasion, inducing cell cycle arrest. This could also potentially promote cellular apoptosis by up-regulating the expression of neogenin (Song et al).

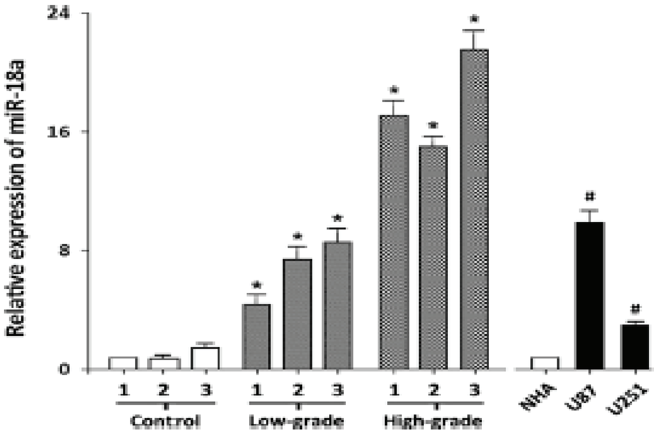

Figure 4 Mir-18a overexpressed in human glioblastoma tissues and cell lines. Assessed in control brain tissues, low-grade glioma tissues, high-grade glioma tissues and NHA, U87 and U251 cells by qRT-PCR.

In conclusion, glioblastoma is a very common type of brain tumor that can arise in anyone. However, it typically arises later on in life. From the studies done above, and studies still being conducted today, researchers are beginning to have a better understanding of glioblastoma. With research continuing, they are able to narrow down and highlight specific areas of chromosomes to use as markers for this devastating cancer. They are also able to identify markers, which maybe in the future will allow for scientists to halt the tumor from growing. This research paper focused on two specific genes that are highly correlated with the disease. From these studies, they are finding how the genes are regulated and how they directly affect how the tumor grows or stops growing. The interesting thing to note is, they now are finding what is correlated with apoptosis of the over expression of the EGFR. If they can pin-point exactly the apoptosis of the EGFR, more options of survival could become more prevalent. However, researchers are looking even deeper into microRNA. It shows how some of the RNA can inhibit or promote growth, along with factors that could one day prevent the tumor from ever growing. The research being done could potentially help future patients who become diagnosed with this horrible cancer. Life expectancy is very short once diagnosed, but with the research being done today, may in turn will have a better outcome for the patients. Better prognosis on how the tumor arises, and how it could potentially be stopped, are big areas of research to come.

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Kelly Kozakiewicz is a recent 2014 graduate and now holds a Bachelor of Science in Biological Sciences. This paper was written for Dr. Ann Green`s Biology Senior Seminar course.