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In silico analysis of wild-type and mutant KRAS

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ABSTRACT

The mutations of the KRAS gene at codons 12, 13, and 61 have been widely reported with different prognosis. In silico is one approach to explain the characteristics of the mutant genes. This study aimed to reveal the potential energy and fluctuations of the binding site and active site of wild-type KRAS (KRAS Wt) and mutant KRAS (KRAS Mt) at codons 12, 13, and 61. The samples used in this study were the sequences of KRAS Wt and KRAS Mt genes, which were subjected to in-silico analysis that included molecular homology, docking, and dynamics using MOE, PyMOL, and online CABS servers. The results showed that fluctuations in the binding site of all KRAS Mt were lower than that of KRAS Wt. On the contrary, the active site (switch I and switch II) of KRAS Mt fluctuated more widely than KRAS Wt. The potential energy of KRAS Mt before forming a complex with GTP was higher ($p<0.01$) than KRAS Wt. After this formation, it remained higher at codons 12 and 61 but lower at codons 11 and 13 ($p <0.001$). Mt G12A did not show any changes. The higher fluctuations in the switch I and switch II regions and the post energy of KRAS-GTP complexes may explain why types of cancers with mutations at codons 11 and 13 have a better prognosis than those with mutations at codons 12 and 61.

Keywords: fluctuations, in silico, KRAS, polymorphism, potential energy

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INTRODUCTION

Abnormalities in cell division are often associated with deviations of proteins involved in cell proliferation and differentiation, which are observable from any conformational changes in the geometric structure.

The Ras Protein and its family, including KRAS, NRAS, and HRAS, are proto-oncogenes, which play roles in transmitting surface receptor signals to effector protein through the PIK3/AKT and RAF/MEK/ERK pathways. These two pathways are responsible for the process of cell proliferation and differentiation. KRAS, NRAS, and HRAS can turn into oncogenes due to mutations in one or more of their base pairs that may increase cell proliferation and differentiation activities and, thereby, trigger resistance to epidermal growth factor receptor (EGFR) monoclonal antibodies (Knickelbein and Zhang, 2014).

Point mutations in the KRAS gene are a type of mutation most commonly found in cancer patients (Karnoub and Weinberg, 2008). The most frequently reported types of cancer that have this gene mutation include pancreatic cancer (up to 70-80%) (Stephen *et al.*, 2014; Garcea *et al.*, 2005), colorectal cancer (up to 40%-90%), lung cancer (30%), breasts cancer (<5%), and fewer types of cancer (de Roock *et al.*, 2010; Forbes *et al.*, 2009). Most of the KRAS gene mutations are localized at codons 12, 13, and 61. However, Miyakura *et al.* (2002) have successfully identified mutation at codon 22, and Hongyo *et al.* (1995) have reported one at codon 11.

Mutations at codon 12 have been reported in up to 80% of all occurrences, while mutations at codon 13 reach 17% and the rest occurs at other codons (Forbes *et al.*, 2006). The KRAS gene mutations in pancreatic and colon cancer have been indicated at codon 12, changing the production of amino acid, that is, the substitution of cysteine for glycine (G12C) (Forbes *et al.*, 2011). In patients with lung cancer, the mutations occur at the same codon as pancreatic and colon cancer, but the alterations in amino acid production involve the conversion of glycine to aspartate (G12D) (Stephen *et al.*, 2014). Also, mutations have been identified at codon 13, i.e., where glycine changes to aspartate, and some other amino acids, and at codon 61 that influences the conversion of glycine to histidine. These mutations often take place in the GDP/GTP bond or called the GTPase domain.

The activities of the GTPase in KRAS Wt and KRAS Mt (G12C, G12D, G12R, G12V, G13D, Q61H and Q61H) have been examined through in vitro analysis by Hunter *et al.* (2015). The results of this analysis showed that KRAS Mt-G12C had the highest GTPase activity, i.e., 72% compared with KRAS Wt. Meanwhile, the GTPase activities occurring in the other mutant forms of KRAS were lower, namely 27.9% in KRAS Mt-G12D, 14.1% in G13D, 6.2% in G12V, 2.6% in G12R, 1.9% in G12A, 1.9% in Q61H, and, the lowest, 1.1% in Q12L compared with KRAS Wt.

The hydrolysis process can be described through the interactions between KRAS Wt and GTPase-activating protein (GAP) complex, specifically between the amino acids Q61 in KRAS Wt, as well as δ and β GTP, with the amino acid R789 in GAP. The NH_2 group of the amino acid R789 in GAP interacts with the CO group of KRAS Wt protein with a bond distance of 2.7 Å. The connection between the NH group of the amino acid R789 in GAP with O (δ and β) atoms in phosphate resulted from hydrolyzed GTP occurs with the bond distances of 2.8 and 3.2 (Å) (Scheffzek *et al.*, 1997). Gao and Leif (2013) also illustrate that the side chain position of Y32 phenol of KRAS Wt protein shifts away from the δ- and β-phosphate of GTP, whereas R789 in GAP is closer to the δ- and β-phosphate of GTP. Y32 phenol group in KRAS Wt is within the distance of 7Å to the R789 in GAP, but it is shorter in KRAS Mt-D31N and D33N (±4 Å). Accordingly, Y32 in KRAS Wt is assumed to play an essential role in opening the gate between this group and the GTP binding site.

According to Futatsugi and Tsuda (2001), the position and orientation of Q61 affect the ability to activate the water molecule 175 (W175). W175, located close to the δ-phosphate in GTP, is considered as a requirement for the hydrolysis process of GTP to GDP. Krengel *et al.* (1990) also confirm this claim after observing KRAS Mt-G12R or G12V mutations. Aside from W175, Lysine 16

has also been reported to be directly involved in the hydrolysis process of the phosphate groups of GTP to GDP when the distances between N atoms (Lys 16) and O1 atoms in the δ-phosphate and β-phosphate of GTP are approximately 3 and 2 Å, respectively (Krengel *et al.*, 1990). Mutations at codons 12, 13, and 61 are believed to cause changes in the orientation and distance of some of these amino acids.

The in vitro test of the activity of the KRAS-GAP complex, including KRAS Wt and KRAS Mt (G12C, G12D, G12R, G12V, G13D, Q61H, and Q61H), has also been carried out by Hunter *et al.* (2015). Based on the test results, the GAP-catalyzed hydrolysis of GTP becomes lower when GTP binds to mutated KRAS. KRAS Mt-G12D exhibited the highest catalytic activity, namely 2.07% activity compared with KRAS Wt. The catalytic activities of the other mutant forms of KRAS varied from 0.12% to 0.74% (0.74% in G12A, 0.56% in G12V, 0.46% in G12C, 0.46% in G12R, 0.46% in G13D, 0.28% in Q61L, and, the lowest, 0.12% in Q61H).

The intrinsic GTPase activity of KRAS and its sensitivity to GAP can also be predicted from other parameters, such as the fluctuations of residues in the GTPase and the two binding motifs in KRAS, namely Switch I and Switch II. Potential energy can also be used in completing the prediction of GTPase activity. The characteristics of these two parameters are useful in assessing the prognosis and healing expectancy of cancer therapy.

MATERIALS AND METHOD

Materials

In this experiment, some programs were used to analyze the mutation of codons. PyMOL v. 0.99 was applied for visualization, MOE 2007.09 for docking and homology modeling, and online CABS flex server for fast simulation of protein structure fluctuations. The data of the sequence of amino acids in KRAS enzyme (Homo Sapiens) was obtained from uniprot.org database (P01116). The KRAS protein template was downloaded from pdb.org (GDP ID: 1WQ1).

Methods

The sequences were transformed according to the mutated amino acids at codons 11, 12, 13, and 61 in FASTA format. The binding sites were identified using MOE, followed by creating KRAS Wt and KRAS Mt enzyme models (A11P, G12A, G12C, G12D, G13A, G13C, G13D, and Q61H). All models were evaluated structurally based on the root-mean-square deviation (RMSD) scores and plots of amino acid residues (Ramachandran Plots) (Petsko and Ringe, 2004). Both ligands and enzymes were prepared and optimized for their three-dimensional structures by adding hydrogen, removing water molecules, adding partial charges, and minimizing energy. Following this procedure was the docking process through the predetermined binding sites. The resulting enzyme-ligand complex was saved in .pdb format, while the docking value was in .mdb. All homology modeling and docking processes were carried out in MOE software.

Data Analysis

The docking results were evaluated by visualization in MOE and PyMol software. Afterward, the analysis continued to the simulation of molecular dynamics for all KRAS before (pre-test) and after (post-test) the formation of the ligand-receptor complex. In this step, MOE was employed to observe potential energy changes, while the CABS-dock web server was used to identify the fluctuations of the constituent amino acids of KRAS Wt/Mt. These data were analyzed statistically.

RESULTS AND DISCUSSION

This research aimed to investigate the potential energy and fluctuations of the binding site and active site of KRAS Wt and KRAS Mt. The results showed that all types of modeled KRAS Mt had 99% structural similarity to the KRAS Wt gene with RMSD<1 Å. Based on the Ramachandran plot analysis presented in Figure 1, all models are considered to have good quality.

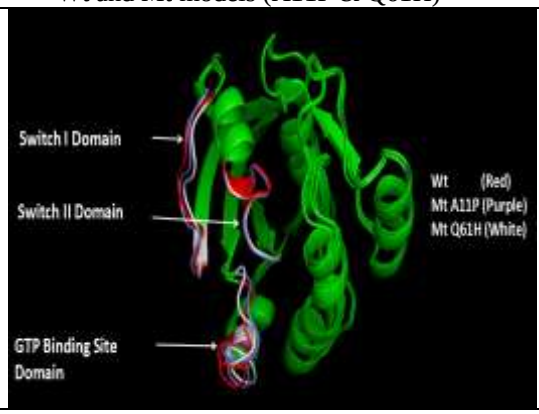
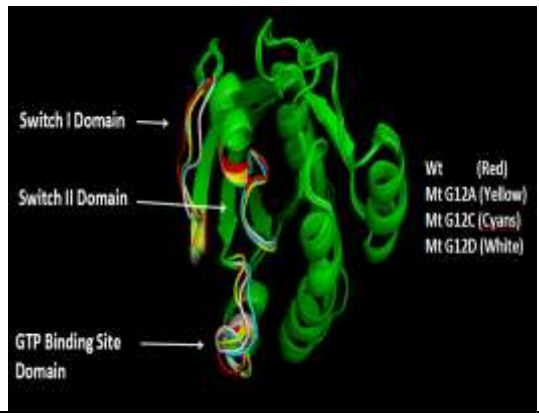
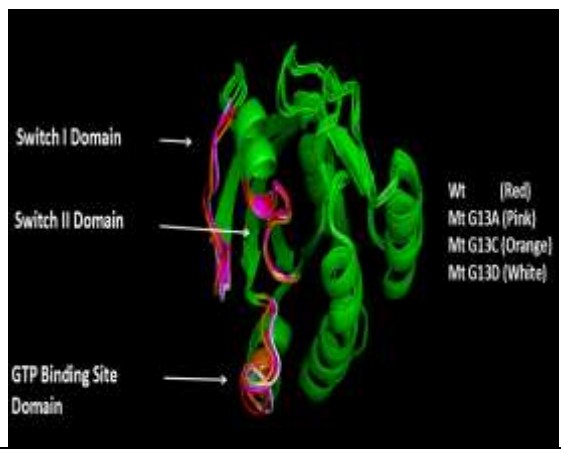
The fluctuations of the binding sites and active sites in the KRAS Wt and Mt models (A11P & Q61H)	Ramachandran plots of the KRAS Wt/Mt models
	1. 94.17% residue in the core region of KRAS Wt 2. 94.51% residue in the core region; RMSD= 0.55 Å (Mt A11P) 3. 94.51% residue in the core region; RMSD= 0.74 Å (Mt Q61H)
	1. 93.90% residue in the core region; RMSD= 0.67 Å (Mt G12A) 2. 93.90% residue in the core region; RMSD= 0.65 Å (Mt G12C) 3. 92.07% residue in the core region; RMSD= 0.59 Å (Mt G12D)
	1. 93.90% residue in the core region; RMSD= 0.55 Å (Mt G13A) 2. 92.07% residue in the core region; RMSD= 0.73 Å (Mt G13C) 3. 92.68% residue in the core region; RMSD= 0.71 Å (Mt G13D)

Figure 1. The fluctuations of the binding site and active site and the Ramachandran plots of the KRAS Wt/Mt models

All models in this experiment meet the requirements of a good-quality structure, namely RMSD is <1 Å and the Ramachandran plot shows >85% of amino acid residues in the core region and <15%

of non-glycine residues in the disallowed region (Petsko and Ringe, 2004). Therefore, all models were subjected to the subsequent analysis.

The KRAS models in Figure 1 show similarities to each other, and the difference mainly lies in the sensitive areas, such as the GTP binding site (codons 11-16), the switch I region (codons 30-38), and the switch II region (codons 60-68). The atomic fluctuations of the GTP binding sites in KRAS Mt and KRAS Wt tended to decrease, as listed in Table I**.**

Table I. Regional fluctuations in "the Domains of the GTP Binding Sites" in KRAS Wt and Mt

Amino Acids	Fluctuations of "the Domains of the GTP Binding Sites" in KRAS Wt/Mt								
	KRAS Wt	A11P	G12A	G12C	G12D	G13A	G13C	G13D	Q61H
11	2.2829	1.5422	2.8341	2.9584	1.8792	2.7353	2.4189	2.4697	3.1452
12	4.1773	3.4073	3.6534	3.8766	2.5942	4.3199	4.385	4.6821	4.656
13	4.9354	4.788	4.4967	4.8156	3.2418	4.181	3.8921	4.4376	4.3667
14	5.6709	4.9264	5.4386	4.2243	3.8444	4.9389	5.0522	4.8114	4.9665
15	1.8831	1.7213	1.5381	1.6565	1.3983	1.7077	1.3699	1.7444	1.5218
16	1.037	0.861	0.8577	0.8916	0.8445	0.7514	0.7871	0.698	0.8127

The decreased residual fluctuations in the GTP binding sites creates a more stable affinity with the ligands. According to Futatsugi and Tsuda (2001), the Lys 16 residue plays a crucial role in forming the KRAS-GTP complex. Table I shows that the fluctuations of the domains of the GTP binding sites in every KRAS Mt model have a downward trend. On the contrary, the changes in the Switch I region of KRAS Mt are more significant than KRAS Wt. The fluctuations of amino acid residues in Switch I have a substantial role in the affinity for and the interaction with GAP (Tyr 32). The data is provided in Table II**.**

Table II. The fluctuations in the Switch I regions of KRAS Wt and Mt

Amino Acids	Fluctuations in the Switch I regions of the KRAS Wt and Mt								
	KRAS Wt	A11P	G12A	G12C	G12D	G13A	G13C	G13D	Q61H
30	12.1551	13.892	15.6219	14.3151	15.6995	14.201	16.1357	12.6599	13.1576
31	12.2375	15.7626	16.8981	15.1176	16.462	14.3461	16.1103	13.8735	14.0005
32	9.658	14.3209	13.6014	12.701	13.1438	12.3517	13.2726	12.1066	13.1425
33	8.5287	11.5732	10.6984	9.7298	10.6493	10.5067	11.0009	10.5711	10.2635
34	6.4181	8.4908	7.8231	7.3217	7.9124	8.2801	8.5336	7.8454	8.1675
35	5.2895	6.3533	5.9763	5.5308	6.1249	6.1802	6.7285	6.1402	5.8718
36	2.726	2.8175	3.0562	3.2252	3.0281	2.8167	3.3429	2.758	2.7221

Table II presents significant fluctuations of Tyr 32 in all KRAS Mt. These fluctuations are assumed to reduce the open conformation of Tyr 32, which allows the insertion of Arginine 789 (amino acid; GAP) and its interactions with δ and β-phosphate (GTP) (Gao and Leif, 2013).

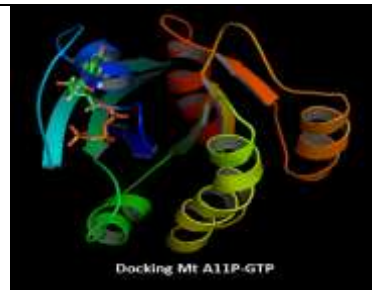
Table III shows that the fluctuations of all amino acid residues in Switch II of KRAS Mt are more significant than KRAS Wt, except for 61 amino acid residues. Variations in these 61 residues in all models (KRAS Wt and Mt) are similar, except for KRAS Mt-G12A and Q61H. Q61H fluctuates almost two times higher than KRAS Wt, which lowers its affinity for and its stable interaction with GAP (Scheffzek *et al.*, 1997). It also reduces the ability of KRAS Mt-Q61H to activate the water molecule 175 that plays a major role in the hydrolysis process of δ-phosphate (GTP) (Krengel *et al.*, 1990). The low interaction between KRAS Mt-Q61H and GAP decreases the catalytic activity of GAP in hydrolyzing GTP (1.9%) (Hunter *et al.*, 2015).

Table III. The fluctuations in the Switch II regions of KRAS Wt and Mt

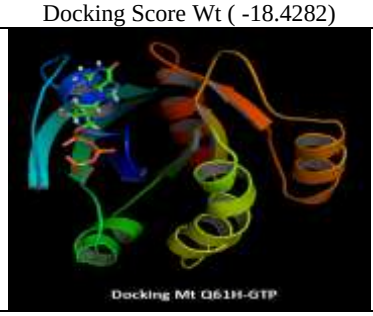
Amino Acids	Fluctuations in the Switch II regions of KRAS Wt/Mt								
	KRAS Wt	A11P	G12A	G12C	G12D	G13A	G13C	G13D	Q61H
60	3.1001	2.1412	2.8756	2.7751	2.3354	2.7159	2.7091	2.7396	2.8013
61	5.2921	4.8806	6.0095	5.2461	4.9861	5.2799	5.2685	5.2171	7.0176
62	8.6949	8.6693	9.0876	8.8353	9.4906	9.1431	9.4787	9.0909	10.1298
63	5.7339	6.891	7.9939	7.8079	8.0632	7.3113	8.6477	7.474	9.9372
64	4.3601	5.9804	5.3523	6.0744	7.2535	6.5767	7.5552	6.4304	6.2479
65	3.0765	5.5553	5.3967	7.061	6.8789	6.1331	9.2252	6.6991	5.1462
66	2.4828	4.3453	4.6005	5.5429	9.9412	5.4177	11.3556	8.7076	4.3489
67	2.1309	4.2736	3.9437	5.4087	9.5005	5.4426	10.4178	8.2599	3.9634
68	1.6122	3.3182	3.1118	3.9449	2.7546	3.1451	3.9649	2.6634	2.8068

The differences in KRAS structures also affect the variations of the ligand-receptor affinity, as observed from the docking scores and the molecular dynamics simulations. The higher the docking score (more negative), the stronger the affinity for GTP (Hardono *et al.*, 2013). Referring to the docking score, KRAS Wt has a relatively weaker affinity compared with KRAS Mt, except for G13C mutant. The ligand-receptor complex formed between GTP and KRAS Mt is stronger, and the signaling function of the messenger is expected to be more active than KRAS Wt. An increase in signaling function leads to uncontrolled cell growth and proliferation. KRAS gene mutation has been reported in almost 30% of cancer cases (Karnoub and Weinberg, 2008). It has also been detected in 70-80% of pancreatic cancers, 40% of colon cancers, and up to 30% of lung cancers (Stephen *et al.*, 2014; Garcea *et al.*, 2005; De Roock *et al.*, 2010; Forbes *et al.*, 2009). KRAS gene mutation is associated with improved signaling function of the messenger.

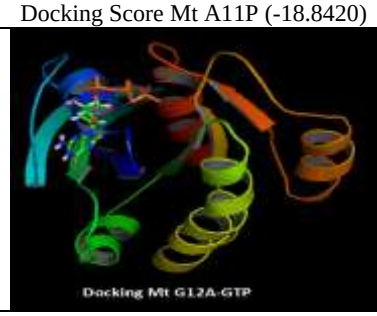
Docking Score Wt (-18.4282)



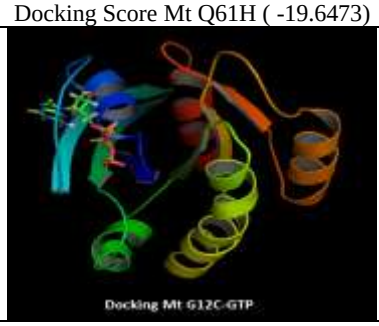
Docking Score Mt A11P (-18.8420)



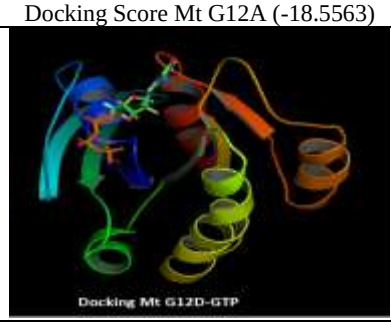
Docking Score Mt Q61H (-19.6473)



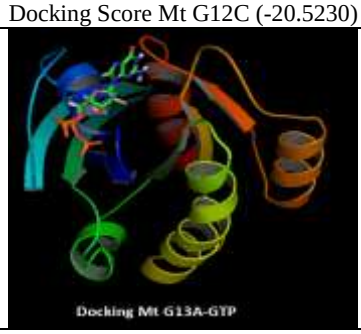
Docking Score Mt G12A (-18.5563)



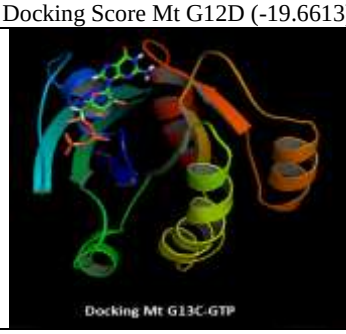
Docking Score Mt G12C (-20.5230)



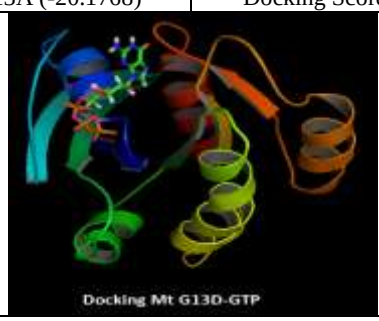
Docking Score Mt G12D (-19.6613)



Docking Score Mt G13A (-20.1768)



Docking Score Mt G13C (-18.1595)



Docking Score Mt G13D (-18.9991)

Figure 2. The results of the KRAS Wt and Mt complex with the docking score of GT

Figure 2 compares the docking results at a specified time interval (0-200 pc) based on the molecular dynamics simulations. Observations were conducted to investigate the potential energy KRAS Mt and Wt before and after the formation of KRAS-GTP complex. The potential energy of KRAS Mt before forming a complex with GTP was significantly higher than KRAS Wt ($p < 0.001$), except for KRAS Mt-G12D and Q61H that had lower potential energy than KRAS Wt. Further analysis with the Tukey's test revealed a very significant difference ($p<0.001$) in KRAS Mt-A11P, G12A, G12C, G12D, and Q61H; in contrast, KRAS Mt-G13A and G13C showed no difference (Table IV).

Table IV. The results of One-way ANOVA of KRAS Wt and all forms of KRAS Mt

Variables	Means	95% CI	*P. Value*	*P. Value of the Tukey's Test*
KRAS Wt	389.90	353.23-426.58		
Mt A11P	496.42	458.95-533.88		0.000
Mt G12A	552.30	515.58-589.03		0.000
Mt G12C	501.03	461.35-540.72		0.000
Mt G12D	298.59	260.56-336.63	< 0.001	0.001
MtG13A	436.01	397.11-474.90		0.088
Mt G13C	424.53	389.15-459.92		0.200
Mt G13D	520.01	483.07-556.95		0.000
Mt Q61H	242.31	203.06-281.55		0.000

The potential energy of the proteins after forming a complex with GTP also showed a very significant difference ($p < 0.001$). Increased energy was identified in Mt G12C, G12D, and Q61H. These mutations cause instability in catalytic activity. As reported in Vatanseyer *et al.* (2017), G12D mutation leads to a shift in the Switch II and α3-helix and this conformational change causes a catalytic disturbance in which the SII movements anti-correlate with other regions. Conversely, decreased potential energy was found in KRAS Wt, Mt A11P, Mt G12A, G13A, G13C, and G13D. Table **V** shows a significant difference between the energy before and after the formation of KRAS-GTP complex ($p < 0.001$), except in Mt G12A ($p > 0.01$). The mutations in KRAS Mt-A11P, Mt G12A, G13A, G13C, and G13D are suspected of exhibiting similar behavior to KRAS Wt. According to Chen *et al.* (2013), KRAS mutations at codon 13 and KRAS Wt have identical behavior. Clinical observations on patients whose tumors have either mutant KRAS-Gly12Cys or mutant KRAS-Gly12Val have worse progression-free survival than those with mutant KRAS proteins or wild-type KRAS in their tumors (Ihle *et al.*, 2012).

Table V. The paired t-test results of the potential energy of KRAS Wt and all KRAS Mt models before and after forming a complex with GTP

Variables	Means	P. Value	N
KRAS Wt	389.91	0.000	201
KRAS Wt-GTP	292.87		
Mt A11P	496.42	0.000	201
Mt A11P-GTP	324.20		
Mt G12A	552.30	0.670	201
Mt G12A-GTP	523.85		
Mt G12C	501.03	0.000	201
Mt G12C-GTP	701.57		
Mt G12D	298.59	0.000	201
Mt G12D-GTP	740.24		
Mt G13A	436.01	0.000	201
Mt G13A-GTP	244.27		
Mt G13C	424.53	0.000	201
Mt G13C-GTP	355.46		
Mt G13D	520.01	0.000	201
Mt G13D-GTP	409.51		
Mt Q61H	242.31	0.000	201
Mt Q61H-GTP	660.09		

CONCLUSION

Increased fluctuations in the switch I and switch II regions and the energy after the formation of KRAS-GTP complexes explain why cancer types with mutations at codons 11 and 13 have a better prognosis than the ones at codons 12 and 61.

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