Project Manual
Bio3055

Lung Cancer:
K-Ras 2

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Lung Cancer: K-Ras2

Introduction:
It is well known that smoking leads to an increased risk for lung cancer, but how does genetics play into the risk? The transformation of a normal cell into a cancerous cell can result from many causes. In one model, factors that lead to an increased rate of mutation in DNA increases the chances that a proto-oncogene will be mutated into an oncogene, causing a normal cell to be transformed into a cancerous cell.

In this module, you will examine one proto-oncogene, K-Ras that has been associated with many cancers, including lung cancer. You will be examining a cDNA sequence for K-Ras that contains a mutation. You can obtain the sequence at the course website under your project name. The file name is shown below:

Saved in FASTA format in file “KRasmutseq”

For your research project, you will analyze the mutant K-Ras protein using the bioinformatics tools presented in lab. You will investigate the structure of the K-Ras protein, model the mutation, and find out what is known, if anything, about the biological impact of the mutation. Through your studies, you will form a hypothesis about what the structural and biological effects are of this mutation, and organize the results of your research into a report. At the last lab session you will present your report to a small group.

Laboratory 1
No Pre-lab assignment
Tutorial on web-based tools

Laboratory 2
Pre-lab assignment:
Complete Reading Questions 1 (page 4). This first reading provides you with a background to genetics and lung cancer. It describes some of the molecular changes that have been linked to lung cancer and how identifying these changes can lead to better predictions of individual risk. You can obtain this article on the course website under your project name.

Readings 1
Laboratory 3
Pre-lab assignment:
   Complete Structure Problem Set (page 6).
   Complete Reading Questions 2 (page 5). This reading provides you with some background for working with the crystal structure of the Ras protein. The pages in the Berg text provide some background about Ras proteins. The article describes the experiment that gave the crystal structure you will be studying as well as how the authors interpreted the crystal structure data. You just need to read an excerpt from this article which is available at the course website under your project name.
      Readings 2
      Excerpt from:

      Berg p. 411 (Section 15.4) to p. 418 (stop at 15.5.1)

Laboratory 4
No Pre-lab assignment
   If you haven’t yet, you should begin preparation for your final report.

Laboratory 5
Pre-lab assignment:
   For this lab, you need to assemble all your research into a report format so you are ready to present your results to the other group working on lung cancer. The other group you will be meeting with has been researching a cytochrome P450 enzyme. Follow the format given in your lab manual for writing the report. At the last lab meeting, you will have 20 minutes to present your findings to the other group. Then they will present their findings. The rest of the lab will be spent working as a group to provide answers to a joint quiz. You will then hand in your reports to be graded.
Questions on Reading 1
K-Ras

Scientific American article: “Uncovering new clues to cancer risk”:

1. What does PAH stand for?

2. Name two sources for PAHs in our environment.

3. Explain how PAH-DNA adducts can lead to cancer.

4. What is some of the evidence presented by the article for associating PAH-DNA adducts with an increased cancer risk?

5. List two types of inherited genetic mutations that may increase cancer risk. Explain how those genes may increase risk for cancer.
Questions for Reading 2
K-Ras

Berg p. 411 – 418

1. What protein is directly upstream of Ras and what protein is directly downstream of Ras in the epidermal growth factor (EGF) signalling pathway?

2. What are the three forms of Ras that mammalian cells contain?

Excerpt from Science article:
In this article, GDP-CP (guanine 5’(-methylene) triphosphate) is a non-hydrolyzable form of GTP (also called a non-active substrate analog).

3. Where are the major structural differences between the GTP-bound and GDP bound forms of Ras?

4. How do the authors explain the interaction between Val at position 12 (Val^{12}) and the gamma phosphate of GDP-CP (the GTP analog)?

5. How does your answer to question 5 explain the increase in activated Ras of the Val 12 mutant?
Structure Problem Set

Directions – Draw the chemical structures for the following amino acids. They are represented in cpk color mode (see Glossary for more information).

1.

2.

3.
4. Draw the chemical representation of the following tripeptide.

5. Draw the chemical representation and represent H-bonds as dotted lines between the atoms where distances have been measured. You will need to add hydrogens that don’t appear in the picture below.

6. What distance must two atoms be in order to be involved in hydrogen bonds and ionic bonds (use the Berg textbook, p. 9 – 10 if needed)?
Guide Sheet 1 Hints and Tips for K-Ras2

Translating the sequence

• Obtain the mutant cDNA sequence from the course website (Krasmutseq)

• Use “Reading Frame 2” when translating the sequence at the Sequence Manipulation Suite.

NCBI – Gene

• Using the Gene database, find the entry for Homo sapiens KRas2. The entry will just say “KRas.” Answer question 1.

• Use the RefSeq entries for the mRNA and protein sequences for K-Ras2 isoform b – also called “variant (b).”

Swiss-Prot Entry

• Use “kras2” to search the SwissProt database

BLAST and ClustalW

• Be sure to choose a good variety of sequences from the BLAST search. The more varied the sequences, the more interesting the alignment will be to study.

• Be sure the wild type human (RefSeq) and mutant sequences only differ by one amino acid residue. If more differences are found, there may have been a mistake in the translation of the mutant sequence.

• Answer questions 7 – 11.
K-Ras2
Questions to accompany guide sheet 1

Gene Entry

1. Fill in the following information from the Gene entry:
   a. Write the GeneID number here ________________.
   b. What is the gene name?
   c. Where in the human genome is this gene located?
   d. What is the RefSeq number for the mRNA sequence for isoform b?
   e. What is the RefSeq number for the protein sequence for isoform b?

Swiss-Prot Entry

2. How many splice variants are there of K-Ras2 and what are they called?

3. Describe how K-Ras2 is activated and inactivated.

4. What proteins does K-Ras2 interact with? (Hint: GDP and GTP are not proteins)

5. Which region is considered “hypervariable” (under “Features”)?

6. Which residue(s) bind GTP (under “Features”)?
Multiple Sequence Alignment

7. What is the mutation in the mutant sequence? Write it in the following format “Res123Res” where the first Res is the three-letter code for the amino acid in the un-mutated (wild type) protein and the second Res is the amino acid in the mutated protein. In place of “123” put the amino acid residue number of the mutation.

8. Is the mutation in a region of conservation?

9. What is the secondary structure predicted for the region containing the mutation?

10. Based on the alignment, what span of amino acids is LEAST conserved? Does this correlate with the region specified in the Swiss/Prot entry as “hypervariable”?

11. What secondary structure is predicted in this area of low conservation?
Guide Sheet 2 Hints and Tips for K-Ras2

Searching for Structure Files: Since the crystal structure of K-Ras has not yet been solved, you will analyze the crystal structure of a closely related protein, H-Ras. The crystal structure of H-Ras complexed with GTP bound (active conformation) has been solved. This is the structure you read about in the reading assignment due for this lab. To obtain the crystal structure data file (pdb file), follow these steps:

1. Go to the Protein Data Bank website www.rcsb.pdb.org (see Glossary) which contains all of the macromolecule 3-D structure files (pdb files). Pdb files are named in 4 characters (numbers and letters).
   a. Search for the 1AGP pdb file. The summary information page for 1AGP contains a title for the entry, the compound crystallized, and the species of the source of the protein. Use this entry to answer questions 1 and 2.
   b. Click on “Download/Display” file at the left of the screen.
   c. On this page, choose to download the structure file in PDB format with no compression. It will be the “none” option in the second table. The “1AGP.pdb” file should now be on your desktop.

Viewing the structure file:

IMPORTANT:
The 1AGP crystal structure was solved with GTP bound in the active site. The H-Ras in this crystal structure is a mutant protein, with Gly12 replaced with an aspartate residue. The GTP is labelled, “GNP 167” in the control panel.

2. Swiss-Pdb Viewer/DeepView has been loaded on your desktop. To open 1AGP.pdb in this program, drag the file to the Swiss-Pdb Viewer/DeepView icon and drop it on the icon. In some cases, double-clicking on the file will also open the pdb file in DeepView.

3. When the file opens, a black screen should appear with the protein shown in wire form. This is a difficult form to view the protein, so we are going to change it to the ribbon form mode. To do this, follow these steps:
   a. First make sure the control panel is open. If you don’t see it, select “control panel” under “Wind”
   b. Click on the control panel window. You can see that all the amino acid residues in the protein are listed in the first column by 3-letter code and residue number. The next columns allow you to change what is displayed. In order to clean up the display of the enzyme, follow these steps:
c. Erase all the check marks in the “show” column and the “side” (meaning side chain) column by clicking on them. For now, you will only view the protein backbone in a ribbon diagram.
d. Put check marks in the “ribbon” column for all the residues.
e. Locate the GNP 167 in the control panel and put a check in the “show” column to show the molecule in the display. The GTP substrate should appear in ball and stick form in the display.
f. Go to the main window and click on the “Display” menu and select “Render in Solid 3-D”. You should now be viewing a ribbon diagram of your protein.
g. You can change the ribbon colors to any color you prefer by selecting “ribbon” under “Prefs”. In this window, make sure the “render as solid ribbon” option (near the top) is selected. You can select different colors for the top, side, and bottom of the ribbons. This allows you to choose a darker version of the same color for the bottom of the ribbon to enhance the 3-D viewing. Take a minute to play around with this option to get the colors the way you would like them. You can also change the background to any color by choosing “Colors” under “Prefs”, then “background”.
h. Click in the display window to make sure that window is the active one. The tool bar for this window is located at the top and is described in your lab manual. Select the “rotate” tool. To rotate the protein, click and hold on the picture while moving the mouse. The other two buttons are “zoom” and “transverse” for zooming in on the protein and for moving the protein from side to side across the screen.
i. Once you have a view that you like of your protein, save it by going to “File” then “Save”. Then select “Layer”. Name your file “hras.pdb” and save to desktop. When you open this file, all your colors and the orientation should be saved, but you will have to select “Render in Solid 3D” again under “Display” to see it. Answer questions 3 and 4.

**Printing the Figure of Your Protein**

4. To save the pdb file as a photo file, we will use the program Grab. You can open Grab by clicking on the scissors icon in the toolbar of your desktop.

5. Make sure the figure is visible exactly the way you want it in Swiss-Pdb Viewer. Then, in Grab, go to “Capture” then “Selection”. You can now draw a box around the part of the view in Swiss-Pdb Viewer that you want to save. Save the file as something ending with “.tiff”. Save it to your
desktop. Now you can now open this file in PowerPoint to use it in a presentation, or Preview to print the figure.

6. Open the “.tiff” file in Preview. Choose Page Setup under “File” and change the scale to 70% to make sure the figure prints on one page. Print a copy of your “.tiff” file.

**Viewing an amino acid side chain**

7. Locate the Asp12 (the same residue location that you are studying) in the structure. Show the side chain by clicking on the “show” and “side” columns in the control panel for that amino acid.

8. Zoom in on this amino acid. Rotate the structure until you can get a good view of the aspartate side chain.

9. Confirm the identity the Asp side chain by using the identity tool on the toolbar and clicking on the side chain in the display window. An atom label should appear with the atom identity and residue number.

10. **The Asp side chain and GTP are colored in CPK mode which is:**
    - red = oxygen
    - blue = nitrogen
    - orange = phosphorous
    - yellow = sulfur and phosphorous
    - gray = carbon
    - light blue = hydrogen

    Take a minute and find the terminal phosphate of the GTP substrate. This is the phosphate farthest from the guanine ring. At this point, you can erase the check marks in the “show” column for all the other residues in order to focus on this side chain and the GTP analog (GNP 167).

**Investigating the environment of the side chain**

In order to investigate the non-covalent interactions of the side chain, use the distance tool on the toolbar to find atoms that are close enough to atoms in the side chain to be involved in H-bonds, ionic bonds, or Van der Waal’s interactions. Keep in mind the atom type when determining what type of interactions may be occurring. The terminal phosphate of GTP will be the focus for identifying non-covalent interactions of the amino acid at the 12 position.

11. Use the distance tool to measure the distance between the nearest oxygen on the aspartate side chain, and the terminal phosphate of GTP.
You will click on the distance tool, then the two atoms that you want to measure the distance between. The distance, in angstroms, should then appear. This step may take several attempts. If you need to erase distances or labels, you can go under “Display” to “labels”, then select “erase user labels.”

Keep in mind the resolution of the crystal structure provides the error in the distances that you are measuring. For example, if the distance is 5 angstroms and the resolution is 2 angstroms, the distance between the atoms is estimated to be 5 angstroms ± 2 angstroms.

12. Once you have identified possible non-covalent interactions involving the Asp side chain, print this view with the distances using the same method as in steps 4 - 6. Save this view as something “.tiff.” You will need the distance measured in step 11 for question 5 of your guide sheet 2 questions.

Modelling the Mutation

13. To change the aspartate side chain to a different side chain, use the “mutate” tool on the toolbar. Select the amino acid cysteine (Cys) to model the K-Ras mutation. The Cys side chain will appear in the lowest energy conformation (most stable). Some green lines may appear which represent potential H-bonds. You can make these disappear under “Display”, then deselect “Show H-bonds”.

14. Use the “distance” tool to measure the distance between the Cys side chain and the terminal phosphate of GTP as you did in step 10 with aspartate. You will click on the two atoms that you want to measure the distance between, then the distance, in angstroms, should appear. Save and print this view following steps 4 -6 and naming the file, “Cys.tiff”. Answer questions 6 – 8.

To put in report:
For this lab, you will need the three figures printed in steps 6, 12, and 14. Make sure the residue numbers and distances are labelled. The distances and labels can be added by hand to the figure if they are difficult to see in the print-out.
Questions to Accompany Guide Sheet 2
K-Ras

1. What organism was H-Ras obtained from for the crystal structure?

2. What is the resolution of this crystal structure and what does “resolution” mean for a crystal structure?

3. Carefully examine the secondary structure in the crystal structure and record any positions where the PSIPRED predictions were incorrect.

4. PSIPRED states its predictions are ~80% correct. Do you agree this is a good estimate of the accuracy?

5. Draw the Asp12 side chain and the GTP molecule from the crystal structure. Include the distances between the atoms you measured in step 10. This should include the correct chemical structure of the aspartate side chain and the phosphate, including atom types, double bonds, etc.
6. Draw the chemical structure of the glycine side chain (the side chain found at position 12 in wild type K-Ras). Would this side chain interact with the terminal phosphate the same as the aspartate side chain does?

7. Analyze the model of the Cys mutation. Can the same non-covalent interaction(s) occur with Asp and Cys? Draw a chemical representation of the Cys residue and the terminal phosphate of the GTP analog. Show any changed non-covalent interactions and distances measured between atoms.

8. What is your hypothesis for how the cysteine mutation affects the activity (on/off state) of K-Ras? Do you hypothesize the mutant K-Ras to be more active, less active or no change from wild type K-Ras (with glycine at position 12)?
Guide Sheet 3 Hints and Tips for K-Ras2

OMIM search

- Search the OMIM database for “Lung Cancer”. Click on the search result that states this exact disease name.

- Scroll down until you get to the list of references. The first article should refer to K-Ras in the title. Click on the PMID# for the first article to bring you to the PubMed entry for this article. The abstract should be displayed. Read the abstract and answer questions 1 – 4.

- Go back to the OMIM entry for lung cancer. At the very top of this entry, the genes linked to this disease are listed. Click on the link listed after KRAS2. This will take you to the OMIM entry for KRAS2.

- Read the first paragraph of the KRAS2 entry and answer question 5. Review the major sections in this entry. An outline for the entry is provided to the left of the window. Go to the Allelic Variants section. Scroll until you see the entry for the Gly12Cys mutation. Answer question 6.

KEGG pathway

Questions to accompany guide sheet 3
K-Ras2

1. Who is the first author on this article and what journal was it published in?

2. Describe who was involved in the study (how many and what categories of patients)?

3. What did the researchers find out about K-Ras mutations?

4. What conclusion(s) did the researchers come to about K-Ras mutations based on their data? (Summarize and put into your own words)
5. Describe the differences and similarities for the K-Ras, H-Ras, and N-Ras genes and proteins.

6. How common was the G12C mutation in the Ahrendt et al. (2001) study?

7. What signaling molecule ultimately leads to Ras activation?

8. What protein(s) is/are directly activated by activated Ras?

9. Elk-1, c-Jun, and c-Fos are transcription factors. In general, what do they act on as a result of Ras activation?

10. If Ras were mutated to be always active, what part of the pathway becomes irrelevant?